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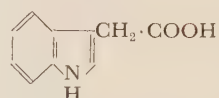
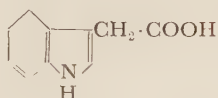
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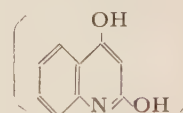
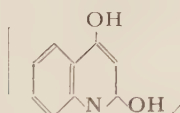
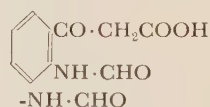
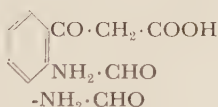
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2	2	18	recovered remained in	recovered in
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40	2	2 (from bottom)	<i>o</i> -Formaminobenzoylactic acid	<i>o</i> -Formaminobnzoylactic acid ethyl estere
41	1	8 (from bottom)	<i>o</i> -formaminobenzoylactic	<i>o</i> -formaminobenzoylacetate
41	1	4 (from bottom)	also decomposition by	also decomposed by
41	2	10 (from bottom)	of the enzyme	of the bacteria
42	(in diagram)			



(add a double-bond at a position of Δ 4, 5)



(add a double-bond at a position of Δ 1, 2)

100	2	11	beginning o	beginning of
158	2	9	pH 3.0,	pH 3.8,
160	1	21 (from bottom)	buffer (5.0).	buffer (pH 5.0).
160	1	13 (from bottom)	32 per cent	20 per cent
160	2	last line (in Table II)	Phenosalfanine	Phenosafranine
220	2	3 (legend of Fig. 5)	A: insulin, pH 12.42.	A: insulin.
220	2	4 (legend of Fig. 5)	B: lysozyme, pH 13.75.	B: lysozyme.
223	2	3	(curve C in Fig. 7)	(curve C in Fig. 5)
223	2	9	curve D in Fig. 7,	curve D in Fig. 5,
243	2 (in Table II)		($-E_{400 m\mu}^{Fe_{ri}}$ /min.)	($-E_{400 m\mu}^{Fe_{ri}}$ /min.)
243	3 (in Table II)		($-E_{400 m\mu}^{Fe_{ri}}$, $E_{280 m\mu}$ /min.)	($-E_{400 m\mu}^{Fe_{ri}}$, $E_{280 m\mu}$ /min.)
249	1	last line	therefore oxidized	therefore oxidized by the oxidase. Cyanide inhibits both the
249	2	14	by the oxidase. Cyanide inhibits both the	(omit)
263	2	4 (from bottom)	ISAMU SHINO	ISAMU SHINO
284	1	14 (from bottom)	(<i>Anthophopleura japonica</i>	(<i>Anthopleura japonica</i>
295	1	25	$C_4H_{12}ON.H_2S_4O_4$	$C_4H_{12}ON_4.H_2SO_4$

Page	Column	Line	For	Read
301	2	2	such pan active	such an active
317	2	1	germinated	germinate
325	2	3 (from bottom)	Ato	A to
329	1	17	The slops, i/n	The slope, 1/n,
329	1	2 (legend of Fig. 2)	the and	and the
380	2	(Fig. 2)		left; (a) right; (b)
410	2	18 (from bottom)	348	353
494	1 (in Table I)	(heading)	μ moles/mg.	μ moles/g.
495	5 (in Table II)	last line	81	8
548	2	21 (from bottom)	3, 5-Diiodo-L-thyroxine(DIT)	3,5-Diiodo-L-tyrosine (T ₂)
551	1	1st line (3rd column in Table III, under T ₀)	0.40	0.30
561	2	7	2000 to 3000g.	200 to 300g.
562	2	9 (from bottom)	minium	minimum
563	1	14	boling	boiling
649	2	3 (from bottom)	atl 40°C	at 140°C
656	2	15 (from bottom)	acetocholeinic	acetocholeic
660	2	9	mainsite	main site
665	1	2	In was	It was
666	2	14	remarkable	remarkable
669	2	8	spstem	system
671	2	16	fraction with	fraction incubated with
683	2	11 (in Scheme I)	H ₁ (just above P ₂)	H
688	1	last line (legend of Fig. 3-A and Fig. 3-C)	6: S ₆	6: S ₅
730	2	1st line (4th column in Table V)	5.72	9.72
744	1	28	40 S	60 S
744	1	29	1.5	1.25
744	1	32	1.5	1.25
748	1	1	linear	Omit "linear"
750	2	5	400Å-100Å	450Å-100Å
754	1	(diagram 7)	$V_s = \sum V_s = \sum \pi r_i^2 d_i$	$V_s = \sum V_s = \sum \pi r_i^2 d'_i$
755	1	38	1.9×10^{-6} in	1.9×10^{-6} cm in
755	2	24	1.5	1.25
756	1	6	$400 \times 100\text{Å}$	$450 \times 100\text{Å}$

The Structure and Function of Ribonuclease T₁

I. Chromatographic Purification and Properties of Ribonuclease T₁

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In Takadiastase have been found two ribonucleases—RNase T₁ (major component) and RNase T₂ (minor component)—with specificity different from pancreatic ribonuclease (RNase I-A). The former has been fairly purified and several enzymatic characteristics have been elucidated by Sato and Egami (1). Of these two, RNase T₁, which specifically hydrolyzes 3'-phosphodiester linkages of guanosine nucleotide in RNA as well as guanosine-2',3'-cyclic phosphate, is of special interest as a favorable material for protein chemistry in connection with its distinct specificity, for this enzyme is a protein of low molecular weight, distinctly stable and relatively abundant in Takadiastase.

Since the crystallization of pancreatic RNase by Kunitz in 1940 (2), several RNases of different origin have been found and studied, but there has been scarcely any investigation on the chemical structure of these enzymes, except the pancreatic RNase (RNase I), which has been extensively investigated regarding its chemical structure and enzymatic function (3) and the complete amino acid sequence has been recently elucidated by the elaborate work of Hirs, Stein and Moore (4) with that of Anfinsen. These results have greatly stimulated the author to elucidate the chemical structure of RNase T₁ for understanding the relationship between the protein structure and the enzymatic function as well as with the interest from the viewpoint of comparative biochemistry.

For this purpose, it has been required to establish a preparative method to supply a sufficient amount of pure enzyme. The pre-

sent paper deals with the chromatographic purification of RNase T₁ on diethylaminoethyl cellulose columns together with slight modifications of the previous procedure and with some physical and chemical properties of the purified enzyme protein.

EXPERIMENTALS AND RESULTS

Determination of Enzyme Activity and Protein

Ribonuclease Activity—The enzyme activity was determined by measuring the optical density at 260 m μ of acid-soluble hydrolysis products with yeast RNA as the substrate as described by Sato and Egami (1) with slight modifications. The reaction mixture contained 0.1 ml. of enzyme solution, 0.25 ml. of 0.2 M Tris buffer, pH 7.5 for RNase T₁ (or 0.2 M sodium acetate buffer, pH 4.5 for RNase T₂), 0.1 ml. of 2×10^{-2} M EDTA aq. solution, 0.3 ml. of distilled water, and 0.25 ml. of RNA (Schwarz preparation, dialyzed and lyophilized, or purified by the procedure described by Frish-Niggemeyer and Reddi (5)) aq. solution (3 mg. per ml.) freshly prepared before use. The hydrolysis was allowed to proceed for 15 minutes at 37° after addition of RNA solution and stopped with 0.25 ml. of 0.75 per cent uranyl acetate in 25 per cent perchloric acid. The reaction mixture was centrifuged and 0.2 ml. of the supernatant solution was pipetted out, diluted with 5.0 ml. of distilled water and the optical density at 260 m μ was read in a Beckmann spectrophotometer model DU, against the blank without enzyme, the reading of the blank lying between 0.1 and 0.2 against water. The enzyme solution was

appropriately diluted before assay—such as 1:10, 1:100, 1:1,000 *etc.*—so that the final diluted solution of 0.2 ml. of the reaction mixture did not give the optical density above 0.400, below which the optical density was in proportion to the enzyme concentration under the above condition.

Protein—Enzyme preparations, both Takadiastase crude extract and the pure enzyme, showed typical absorption spectra of protein with a maximum at around $280\text{ m}\mu$, and so the protein was determined by the absorption at this wave length, assuming that 1 mg. protein in 1 ml. showed the optical density of 1.000 at $280\text{ m}\mu$ for convenience' sake and for economy of the sample. The results showed a good proportionality to those obtained by the method of Folin-Ciocalteu.

Definition of Enzyme Unit and Specific Activity—The amount of the enzyme that caused the increase in the optical density at $260\text{ m}\mu$ by 1.000, as corrected by dilution factor, under the specified condition was defined as one enzyme unit. Total enzyme units in a sample solution was (enzyme units in 0.1 ml. of appropriately diluted sample solution determined under the given condition) \times (dilution factor) \times (volume of the initial sample solution in ml. $\times 10$). The specific activity of the enzyme preparation was defined as the ratio of the enzyme units in 0.1 ml. of the sample solution divided by the optical density at $280\text{ m}\mu$ of the same solution or (total units of the sample/protein (g.)) $\times 10^{-4}$.

Purification of RNase T_1

Partial Purification

The enzyme was partially purified before chromatographic purification according to the procedure of Sato and Egami (1), with some modifications. One kg. of Takadiastase-Sankyo (containing about $3,000 \times 10^3$ units of RNase T_1 , which differed from each other a few fold by lot) was extracted with 4 liters of water and the extract was heat-treated in twenty separate portions at 80° for two minutes at pH 2.5 with vigorous stirring and quickly cooled to room temperature. The heat-treated extract was filtered and the bulky residue

was extracted with water to recover the remaining enzyme. In case the extraction of Takadiastase was carried out with 2 liters or less of water, larger part of the enzyme was found remaining in the residue. The combined filtrate adjusted to pH 6.0 was then added with solid ammonium sulfate to 0.6 saturation and kept to stand for several hours. The supernatant solution was filtered and brought to saturation at pH 4.0. As shown in Fig. 1, RNase T_1 and T_2 were successfully separated, but the precipitation of RNase T_1 was not satisfactory at saturation at pH 6.0 and about 40 per cent of the enzyme remained in the supernatant. At pH 5.8, about 65 per cent of the activity at pH 7.5, about 80 per cent of the activity at pH 4.5 and about 40 per cent of the protein were recovered remained in the precipitate between 0 and 1.0 saturation. At lower pH, the enzyme became more precipitable as shown in Fig. 2 and so the satura-

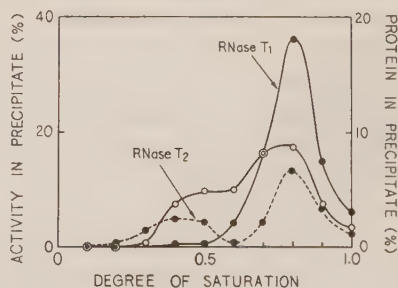


FIG. 1. Ammonium sulfate fractionation of heat-treated RNase T_1 preparation. Heat-treated RNase T_1 preparation (1.1×10^3 units) was fractionated at pH 5.8 into ten fractions and the precipitates formed between each of saturations were collected and the activities (at pH 7.5 —●—, and pH 4.5 ---●---) and the protein (—○—) were determined. The initial activity at pH 7.5 and protein of the preparation before ammonium sulfate fractionation were taken as 100 per cent. The activity at pH 4.5 denotes the relative value to that at pH 7.5.

tion at pH 4.0 was preferred to obtain much higher yield. The precipitate was extracted and dialyzed against cold water and subjected to the second ammonium sulfate fractionation under the same condition given above. The extract of the precipitate was exhaustively

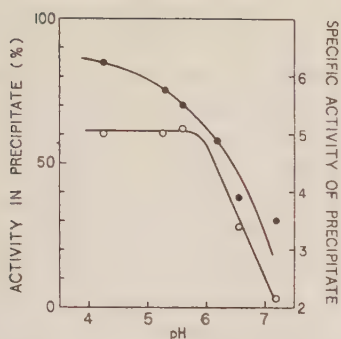


FIG. 2. Ammonium sulfate fractionation at various pH values. RNase T₁ preparation (supernatant solution at 0.6 ammonium sulfate saturation at pH 6.0, 5.5×10^3 units, specific activity: 1.1) was brought to saturation at various pH values, adjusted with 2 N NaOH or 2 N HCl, and the precipitates were collected, extracted with 0.1 M sodium citrate buffer of pH 6.0 and the activity was determined. —●—: enzyme activity, —○—: specific activity.

dialyzed against cold running tap water overnight and stored deepfrozen or further subjected to calcium phosphate gel treatment. At this stage, about 40- to 60-fold purified enzyme was obtained in 33 to 43 per cent overall yield.

Chromatographic Purification on DEAE-Cellulose:

General Procedure—The preparation of DEAE-cellulose columns and the chromatographic procedure were carried out according to the general procedure of Sober *et al.* (6). About 20 g. of DEAE-cellulose (Eastman preparation) was washed with 2 liters of 1 N NaOH and then with large volume of water until the washings were colorless and the pH, below 10. The exchanger was packed into a column under gravity and washed with about 1 liter of 0.005 M Na₂HPO₄. Twenty grams of the exchanger were sufficient to give four to five columns of 1.5×15~20 cm. or two columns of 2.5×15~20 cm. The exchanger after use was regenerated by the same procedure given above and reused.

The dialyzed enzyme solution was added with one-hundredth volume of 0.5 M Na₂HPO₄ (to a final concentration of 0.005 M, pH around 7) and loaded on a column of DEAE-

cellulose equilibrated with 0.005 M Na₂HPO₄. The chromatography was carried out by a gradient elution system—the mixing chamber of 600 ml. was filled with 0.005 M Na₂HPO₄ and the upper chamber of 1 liter, occupied with 0.25 M NaH₂PO₄ containing 0.25 M NaCl. A 1.5×20 cm. column could be loaded as much as 2 g. protein, the moderate load being 1 g. or less. The elution rate was usually controlled to 15~20 ml. per hour, which could be increased five-fold without much effect. The effluent was collected in 5 or 10 ml. fractions with an automatic fraction collector and the enzyme activity and the protein were measured as described above. The chromatography was completed within two days.

The chromatography was also carried out with a similar result on a column equilibrated with 0.005 M sodium phosphate buffer of pH 6.7 by a salt gradient system, in which the 600 ml. mixing chamber was filled with 0.005 M sodium phosphate buffer of pH 6.7 and the upper chamber was occupied with the same buffer containing 0.5 M NaCl.

Chromatography of Takadiastase Extract—As a preliminary experiment, crude extract of Takadiastase with water was directly chromatographed and the behavior of RNase T₁ and T₂ on the column was observed. Five hundred milligrams of Takadiastase was extracted twice with 2.5 ml. of distilled water, diluted to 20 ml. with distilled water and loaded on a column of 1.5×15 cm., after adjusting the pH of the extract to 7.0. The chromatogram is shown in Fig. 3.

The RNase activity was separated into four peaks—one major and three minor peaks. The major peak, IV, was that of RNase T₁, peaks II, and III, which were more active at pH 4.5 than at pH 7.5, corresponding to so-called RNase T₂ and the pass-thru peak, not yet characterized RNase or phosphodiesterase. The chromatogram showed that more than ninety per cent of the RNase activity in Takadiastase was ascribed to that of RNase T₁, though the ratio of the components differed somewhat by lot, down to eighty per cent in RNase

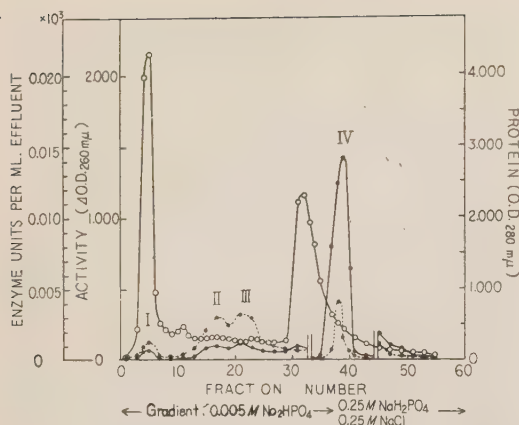


FIG. 3. Chromatogram of Takadiastase crude extract on a column of DEAE-cellulose. Column: 1.0×15 cm. Load: water extract of 500 mg. Takadiastase (4.7×10^3 units). Elution: gradient from $0.005 M Na_2HPO_4$ to $0.25 M NaH_2PO_4$ (containing $0.25 M NaCl$) with a 600 ml. mixing chamber, at the flow rate of $15 \sim 20$ ml. per hour. Fraction: 10 ml. per fraction. Activity: optical density at $260 m\mu$ of acid-soluble hydrolysis products from yeast RNA by 0.1 ml. of the effluent was measured at pH 7.5 (—●—) and 4.5 (---●---); the effluent fractions between no. 33 and 43 were assayed after ten- or 100-fold dilution and the activities of these fractions are given in 1:10 scale in the figure. Protein: measured by the optical density of each of the effluent fractions (—○—).

T_1 content. In this chromatography, about ten- to thirty-fold purified RNase T_1 , free from RNase T_2 , could be obtained in about 80 per cent yield. Rechromatography of the RNase T_1 fraction gave more than 100-fold purified preparation. Under the above condition, the load could be increased up to ten-fold with good reproducibility.

Chromatography of Partially Purified RNase T_1

—The direct chromatography of Takadiastase crude extract was not of practical use for large-scale preparation from kg. quantities of Takadiastase. For this purpose were employed the partially purified RNase T_1 preparations obtained by the ammonium sulfate fractionation or those further subjected to the gel-treatment as described above or elsewhere (1). In Fig. 4 are given chromatograms of these preparations. About 50- to 60-fold purified RNase T_1 preparation of 200

$\times 10^3$ to 400×10^3 units (protein: $0.5 \sim 1.5$ g.) was loaded on a column of 1.5×20 cm. or that of 600×10^3 to 1000×10^3 units on a column of 2.5×20 cm. and after passage of relatively large amount of pass-thru protein, the elution was carried out under the specified condition. RNase T_1 was adsorbed at the column top in dark-brown band and eluted rather retarded in the effluent between 350 and 500 ml. with good reproducibility. In contrast to the sharp activity curve, the protein was not sharply eluted; greater part of the protein was eluted before emergence of RNase T_1 and as is clear in Fig. 4, the protein separable by the gel-treatment was largely removed from the enzyme on the column without this treatment.

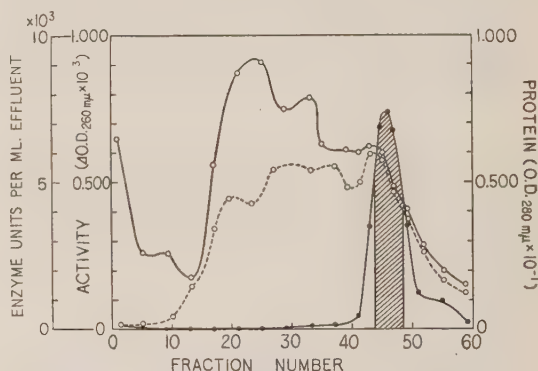


FIG. 4. Chromatograms of partially purified RNase T_1 on a column of DEAE-cellulose. Column: 2.5×20 cm. Load: about 700×10^3 units of RNase T_1 preparation partially purified by ammonium sulfate fractionation (specific activity: about 10, protein: about 7 g.) and that further purified by the gel-treatment (specific activity: about 30, protein: about 2.7 g.) respectively. Fraction: 10 ml. per fraction. Activity: determined under the specified condition after 1000-fold or 2000-fold dilution and shown in the figure in 1:1000 scale (—●—). Protein: determined after 10-fold dilution and shown in 1:10 scale. —○—: preparation purified by ammonium sulfate fractionation; ---○---: preparation further purified by the gel-treatment. The shadowed fractions were pooled and subjected to rechromatography.

In this chromatography, about 300- to 400-fold purified enzyme preparation was obtained in 80 to 90 per cent yield. In case the starting preparation was less than 20-fold purified (specific activity below 10), about 200- to 250-fold purified enzyme was obtained. The RNase T_1 fractions of specific activity

above 100 were combined and dialyzed against cold distilled water overnight. At this stage, the enzyme solution was not required to be thoroughly dialyzed but to the concentration below 0.1 *M*, under which the enzyme was firmly bound to the DEAE-cellulose. Upon extensive dialysis of the enzyme solution of relatively high concentration against water, the enzyme was partly precipitated and much loss occurred from the supernatant. In this occasion, the enzyme in the precipitate was extracted with 0.1 *M* sodium phosphate buffer of pH 7.0.

Rechromatography of RNase T₁—The enzyme preparation obtained in the preceding chromatography was subjected to rechromatography under either of the given elution condition. As shown in Fig. 5, RNase T₁ was eluted as a single peak with respect to the enzyme activity as well as to the protein and about

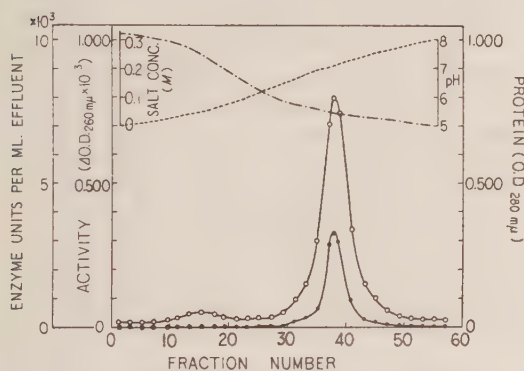


FIG. 5. Rechromatogram of RNase T₁ preparation on a column of DEAE-cellulose. Column: 1.5 × 20 cm. Load: about 200 × 10³ units of RNase T₁ preparation obtained in the first chromatography (specific activity: 150~200; protein: 100~130 mg.). Fraction: 10 ml. per fraction. Activity: determined after 1000-fold dilution and given in 1:1000 scale, —●—. Protein: determined without dilution, —○—. (----: salt concentration, — — — pH).

700- to 800-fold purified RNase T₁ (specific activity: 370~400) was obtained in 80 per cent yield. In some cases, the effluent solution in the first chromatography was three- to four-fold diluted with distilled water, adjusted to pH around 7 with 2 *N* NaOH

and directly loaded on the column without the preceding dialysis to avoid the loss during dialysis and for economy of time, when almost the same purification was achieved with higher yield.

In case the enzyme preparation obtained in the first chromatography was less than 200-fold purified, the second chromatography often provided about 500- to 600-fold purified enzyme, which was further purified by repeating the chromatography once more under the same condition.

The most purified preparation obtained in the rechromatography was shown to be a single component and the specific activity was not appreciably raised in further chromatography under modified elution conditions—the 600 ml. mixing chamber was occupied with 0.005 *M* Na₂HPO₄ and the upper chamber with 0.25 *M* NaH₂PO₄; the former with

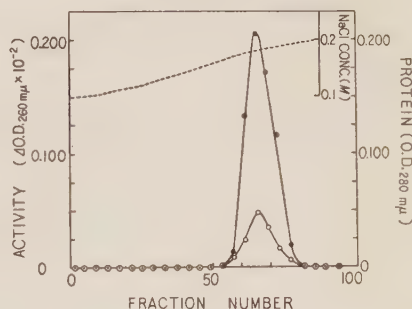


FIG. 6. Chromatogram of the most purified RNase T₁ on a column of DEAE-cellulose. Column: 1.0 × 15 cm. (equilibrated with 0.005 *M* sodium phosphate buffer of pH 6.7 containing 0.1 *M* NaCl). Load: about 4 mg. of RNase T₁ (24 × 10³ units) purified by column-chromatography on DEAE-cellulose. Elution: gradient from 0.1 *M* to 0.25 *M* NaCl in 0.005 *M* sodium phosphate buffer of pH 6.7 with a 600 ml. mixing chamber. Fraction: 6.4 ml. per fraction. Activity: determined after 100-fold dilution and given in the figure in 1:100 scale, —●—. Protein: determined without dilution, —○—. NaCl concentration, ----.

0.005 *M* sodium phosphate buffer of pH 6.7 and the latter with 0.25 *M* NaCl in the same buffer or the former with 0.1 *M* NaCl in 0.005 *M* sodium phosphate buffer of pH 6.7 and the latter with 0.3 *M* NaCl in the same

buffer as shown in Fig. 6.

The enzyme fractions were combined, dialyzed exhaustively against several changes of cold distilled water and lyophilized. The lyophilization often caused slight inactivation of the enzyme. The purified enzyme was obtained in almost colorless state in the yield of about 100 mg. from 1 kg. Takadiastase (about 20 per cent overall yield) and stably stored in a desiccator over calcium chloride in a deep-freezer or in a refrigerator. The results of a typical preparation of this enzyme are summarized in Table I.

TABLE I
Purification of RNase T_1 from 1 kg. Takadiastase

Stage	Protein ¹⁾ (g.)	Total units ($\times 10^3$)	Specific activity ²⁾	Activity yield (%)
Crude extract	500~600	3,000	0.5~0.6	100
Heat-treatment	200~300	1,500~ 2,000	0.7~1.0	55~66
Ammonium sulfate fractionation (twice)	5~6	1,000~ 1,300	20~30	33~43
First chromatography	0.4~0.6	800~ 1,000	150~ 200	26~33
Second chromatography	0.16~0.2	600~ 800	370~ 400	20~27

1) Protein was estimated from the optical density at 280 m μ for convenience' sake and the value for the most purified preparation should be multiplied by 1/1.67 to obtain the actual amount (g.) of the enzyme.

2) Specific activity is expressed as (total units $\times 10^{-4}$) / (protein (g.)).

Properties of RNase T_1

The purified preparation was shown to be homogeneous by sedimentation, paper-electrophoresis and N-terminal amino acid analysis.

As detailed in the part of Addendum (7), the purified enzyme showed a single symmetrical boundary of sedimentation constant 1.62 S on the ultracentrifugal analysis and the molecular weight was determined to be 11,000 (f/f_0 : 1.21) as calculated from the sedimentation coefficient and the diffusion coefficient, 12.0×10^{-7} cm.² per sec.

Upon paper-electrophoresis at pH 6.7, the enzyme moved towards the anode with the rate of about 2.7×10^{-5} cm./sec. per volt/cm. and showed a single symmetrical peak as shown in Fig. 7. Under the same condition, RNase I moved slightly towards the cathode. The electrophoresis was also carried out under other conditions to give a single peak, though the enzyme moved only slightly at pH below 5.0 and hardly moved at pH 3.5 or below.

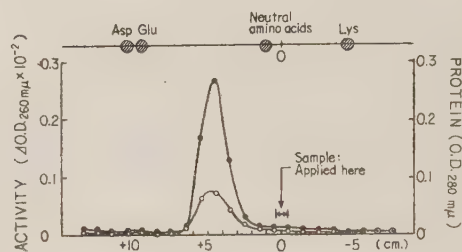


FIG. 7. Paper-electrophoretic pattern of RNase T_1 . Load: one mg. RNase T_1 (6.1×10^3 units) was applied onto a filter-paper of Toyo-Roshi No. 51, 3.3×30 cm. Electrophoresis: 300 volts/30 cm., 2~2.3 mA, 0°, 5.5 hours, in 0.2 μ sodium phosphate buffer of pH 6.7. Activity and protein: the paper after electrophoresis was cut off by 1 cm. width, eluted with the above buffer to 4 ml.; the activity was determined after 100-fold dilution, —●—, and the protein, without dilution, —○—. Some amino acids were run at the same time under the same condition, and the positions were detected with ninhydrin reagent.

The N-terminal amino acid analysis was carried out according to the procedure of Sanger (8) and Levy (9). The lyophilized enzyme (7.21 mg, 0.654 μ moles) was dinitrophenylated in 66 per cent ethanol (1.8 ml.) containing 4 mg. dinitrofluorobenzene and 40 mg. NaHCO₃ at 37° for 12 hours. The acid hydrolysis was carried out in 1 ml. of 6 N hydrochloric acid at 105° for 6 hours. The ether-soluble DNP-amino acids were subjected to two-dimensional paper-chromatography, using *n*-butanol saturated with 2 N NH₄OH as the first solvent and 1.5 M sodium phosphate buffer, pH 6, as the second solvent. In this chromatography, only a single spot of DNP-amino acid was obtained at the position

of R_f 0.5 (butanol-2*N* NH₄OH) and 0.24 (1.5 *M* Na-phosphate buffer), corresponding to DNP-alanine. The water-soluble DNP-amino acids were chromatographed using butanol-acetic acid-water (4:1:5 vol./vol.) as the developing solvent after desalting through a talcum obtained column. A single spot was obtained at the position of ϵ -DNP-lysine (R_f : 0.63), and was negative to the Sakaguchi reaction, indicating the absence of DNP-arginine. The two DNP-amino acids were subjected to spectrophotometry in *N* HCl, each showing at typical spectrum with the absorption peak at 353 m μ corresponding to DNP-alanine and that at 363 m μ corresponding to ϵ -DNP-lysine respectively. The recovery was 0.067 μ moles for DNP-alanine and 0.10 μ moles for ϵ -DNP-lysine without correction owing to the loss through the procedure.

The enzyme showed a typical protein spectrum in the ultra-violet region with the maximum absorption at 278~279 m μ (O.D.₂₇₉/O.D.₂₆₀: 1.45) and the enzyme solution of one mg. per ml. showed 1.6₇ in the optical density at this wave length. One mg. of the enzyme possessed 6.1×10^3 activity units and the specific activity was about four-fold higher than

DISCUSSION

For the structural study of the enzyme, it is especially important to obtain pure enzyme in good yield. RNase T₁ is an enzyme protein of acidic nature and cation-exchangers such as carboxymethyl cellulose and IRC-50 which were successfully applied to purification of basic proteins such as RNase I and lysozyme, were found to be of little value for the purification of RNase T₁, though some purification was achieved by displacement chromatography on a XE-64 column equilibrated with 0.01 *M* sodium citrate buffer, pH 3.0 and elution with 0.1 *M* sodium citrate buffer, pH 4.5. On the contrary, DEAE-cellulose, an anion-exchanger, was successfully applied to the chromatographic purification of this enzyme. One kg. of Takadiastase was found to contain about 500 mg. of RNase T₁ on an average, the content differing somewhat by lot. In the direct chromatography of Takadiastase crude extract on DEAE-cellulose, purification was somewhat achieved, but it was hardly of practical use for large-scale preparation as from kg. quantities of the starting material, though this simple procedure will find use for small-scale preparation of RNase T₁ and RNase T₂ for enzymatic use.

For large-scale preparation, partial purification—heat-treatment and ammonium sulfate fractionation—was required to be made before chromatographic purification. Upon heat-treatment, an appreciable amount of loss of the enzyme occurred and so this step seems to have room for re-examination to obtain higher yield. The precipitation of the enzyme at ammonium sulfate saturation at pH 4.0 instead of that at pH 6.0 appreciably increased the yield, which will be related to the low isoelectric point of this enzyme. The calcium phosphate gel treatment seems not necessarily to be required, for almost the same extent of purification was achieved in the subsequent chromatography with or without this step. The fact that the enzyme was firmly bound to the exchanger

TABLE II

Physical and Chemical Properties of RNase T₁

Sedimentation constant ($S_{20,w}$)	1.62 S (pH 6.50)
Diffusion constant ($D_{20,w}$)	12.0×10^{-7} cm ² per sec
Molecular weight	11,000
Frictional ratio (f/f_0)	1.21
Electrophoretic rate	2.7×10^{-5} cm/sec per volt/cm (pH 6.7, 0.2 μ sodium phosphate buffer)
Absorption maximum	278~279 m μ
O.D. ₂₇₉ /O.D. ₂₆₀	1.4 ₃
O.D. _{1 cm} ^{0.1%}	1.6 ₇
Enzyme activity	6.1×10^3 units per mg.
N-terminal amino acid	alanine

that of RNase I as measured under the above-mentioned condition. Some physical and chemical properties are listed in Table II.

below the salt concentration of $0.1M$ and eluted much retarded from the column above $0.17M$ indicates strong acidity of the enzyme, as was shown by the paper-electrophoresis. This acidity enables the enzyme to be concentrated from large volume of dilute enzyme solution. The precipitable property of this enzyme during dialysis against water seems to indicate its globulin-like nature. The results of chromatography, sedimentation, paper-electrophoresis and N-terminal amino acid analysis may be sufficient to demonstrate the homogeneity of the purified enzyme.

SUMMARY

1. A method for large-scale preparation of pure RNase T_1 from Takadiastase by column-chromatography on DEAE-cellulose was described with some modifications of the previous procedure.

2. The purified preparation was demonstrated to be homogeneous by chromatography, sedimentation, paper-electrophoresis and N-terminal amino acid analysis.

3. A molecular weight of 11,000 was obtained, based on the sedimentation and diffusion coefficients.

4. The N-terminal amino acid was de-

termined as alanine.

The author wishes to express his sincere thanks to Prof. F. Egami for his guidance and encouragement during this work and to Prof. N. Ui for the ultracentrifugal analysis and determination of the molecular weight of RNase T_1 . He also expresses his gratitude to Sankyo Co. Ltd. for the kind gift of "Takadiastase Sankyo". The expense of this study was defrayed in part by a grant from the Ministry of Education.

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ADDENDUM:

Sedimentation and Diffusion of Ribonuclease T₁

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(Received for publication, June 21, 1960)

The chromatographically purified preparation of ribonuclease T₁, described in the preceding paper (1), was examined by sedimentation and diffusion in order to assess the degree of homogeneity as well as its molecular weight.

EXPERIMENTAL

The lyophilized sample of ribonuclease T₁ was dissolved in a phosphate buffer of pH 6.50 and ionic strength of 0.2, and the solution was subjected to sedimentation after appropriate dilution if necessary. Prior to diffusion experiment, the solution was dialyzed against the same buffer for two days at a low temperature. Protein concentrations were determined spectrophotometrically at 280 m μ .

Sedimentation experiments were conducted in a Spinco model E ultracentrifuge at 59,780 r.p.m. using a synthetic boundary cell. Temperature of the solution was determined during each run using a rotor-temperature indicating unit. The observed sedimentation coefficients were always corrected to the values in water at 20° (*s*_{20, w}) in the conventional manner.

The diffusion measurement was made with a 0.19 per cent solution at 20.0° in a Neurath-type diffusion apparatus (2) equipped with a Philpot-Svensson's schlieren optical system (3). Diffusion coefficient was computed from the second moments of the diffusion curves (4) and corrected to the value in water at 20°.

RESULTS AND DISCUSSION

As shown in Fig. 1, the ultracentrifugal patterns of ribonuclease T₁ showed only a single symmetrical boundary indicating its homogeneity. The calculated value of the sedimentation coefficient was found to be independent of the protein concentration (Table I), and a value of 1.62S was taken as the mean value.

The diffusion curves almost coincided with a Gaussian curve. The view that this preparation is homogeneous was thus supported. The diffusion coefficient was calculated to be 12.0 $\times 10^{-7}$ cm²/sec.

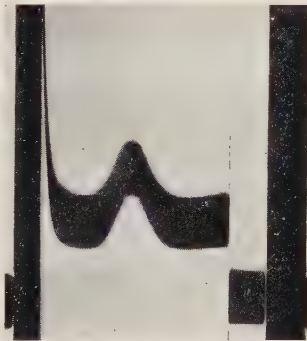


FIG. 1. Sedimentation pattern of ribonuclease T₁, 40 minutes after reaching the speed of 59,780 r.p.m. Sedimentation proceeds from right to left. Cell: synthetic boundary cell. Solvent: phosphate buffer, pH 6.50, ionic strength 0.2. Concentration: 0.91 per cent.

TABLE I

Sedimentation Coefficients of Ribonuclease T₁ in a Phosphate Buffer of PH 6.50 and Ionic Strength of 0.2

Concentration (%)	Temperature	<i>s</i> _{20, w} (S)
0.91	19.9°	1.62
0.69	21.0°	1.67
0.46	20.8°	1.63
0.23	21.1°	1.57
mean	...	1.62

The molecular weight and frictional ratio were calculated on the assumption that the

partial specific volume of this preparation is equal to the value of pancreas ribonuclease, *i. e.*, 0.709 cc./g. (5, 6) which is not far from the value of partial specific volume (0.697 cc./g.) calculated from the amino acid composition of ribonuclease T₁*. A value of 11,000 was obtained for the molecular weight, which seems a little lower than the reported value of pancreas ribonuclease (12,700—13,895) (5-7). The frictional ratio obtained, 1.21, suggests that the molecule of ribonuclease T₁ is more elongated or more solvated than the pancreatic one.

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Studies on Phospholipids

I. Comparative Analysis of Phospholipids from Mammalian Blood Stroma and Spleen

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(Received for publication, July 8, 1960)

A number of studies on the constitution of phospholipids of various animal tissues has been reported. However, no satisfactory method for their quantitative determination has ever been established. Most of the assay methods reported so far are based on indirect procedures, because of the lack of the method for the separation of phospholipids.

Relatively large progress has been made in the research of sphingomyelin and glycolipids of blood stroma (1-11), but only a little information has so far been found about its lipids which are soluble in ether.

Ratio of these phospholipids in erythrocytes of mammals especially of human beings has been reported by several workers (12, 13, 14). However, the results are not in agreement with each other.

It was found that erythrocytes of ruminants such as cattle, sheep and goat contained a very small amount of lecithin (15, 16, 17), and although this finding could explain to some extent the serologically specific behaviour of these red cells, the more detailed analyses of the phospholipid composition of these cells have been desired. Very few reports were found on the presence of inositol in mammalian red cells (18). In view of a very rapid turnover of diphosphoinositide in guinea pig brain (19) and of the presence of phytic acid in avian red cells (20), it was thought necessary to carry out a determination of the content of inositol in erythrocytes prior to the investigation of its role.

In the present study, some attempts have been made in comparative biochemistry of

phospholipids from red cells of various animals applying the recently developed chromatographic method for separating phospholipids on silicic acid column or silicated paper, and the ratio of phospholipids and inositol content in red cells was determined for each animal species.

MATERIALS AND METHODS

Phospholipids of Erythrocyte Stroma—Preparation of erythrocyte stroma and extraction of phospholipids from them were carried out by the prescribed method (1). Lipids fractionated into ether-soluble and -insoluble fractions in the cold, the former being passed through a cellulose column before use (21).

Horse and Bovine Spleen Phospholipids—The tissue was homogenized and dehydrated with acetone, air-dried, and extracted repeatedly with a mixture of ether and methanol (1:1) at room temperature. After removal of the solvents and precipitation with acetone, the precipitate was fractionated into ether-soluble and -insoluble fractions and the former was passed through a cellulose column before use.

Materials—Materials thus obtained were all stored in ether at 0°C.

Analytical Methods—Nitrogen was determined by the micro-Dumas method, phosphorus by Allen's method (22), ester linkage by ferric hydroxamate procedure of Snyder *et al.* (23), choline by Appleton's method (24), ethanolamine and serine by FDNB method (25), and glycerol by Blix' method (25). Amino-nitrogen of intact phospholipid was estimated by the ninhydrin method of Lea *et al.* (27). Inositol was assayed turbidimetrically using *Saccharomyces carlsbergensis* (28).

General Procedure of Column Chromatography—Columns of 11, 16, 22 and 32 mm. in diameter were used. A typical experiment was as follows: A mixture of

silicic acid (50 g., Mallinckrodt's reagent grade, 100 mesh) and Hyflo Super Cel (25 g.) slurried with chloroform (previously contained 1 per cent of methanol) was added into a column, 22 mm. in diameter. After the mixture of silicic acid and Hyflo Super Cel settled down, a phospholipid sample dissolved in the same solvent was loaded on to the column in an amount less than 1 mg. of lipid-P per 1 g. of silicic acid, and the elution was begun with chloroform. After 200 ml. of the solvent was run, the column was successively eluted with 400 ml. each of chloroform containing 5, 10, 15, 20, 25, 30, 35, 40 and 60 per cent methanol, and finally with an equal volume of methanol. Fractions were collected in 40 ml. portions and aliquots from these fractions were submitted to estimations of phosphorus and ninhydrin coloration.

Paper Chromatography of Phospholipids on Filter Paper Impregnated with Silicic Acid—Impregnation of paper with silicic acid generally followed the procedure of Lea *et al.* (29). In the present work, Toyo filter paper No. 53 (40×40) was used. The filter paper was immersed in the solution of sodium silicate (300 g. of silicic acid (Mallinckrodt) dissolved in 2400 ml. of sodium hydroxide (220 g.) solution) for five minutes, drained for five minutes, and treated with 6*N* HCl for 30 minutes. The filter paper was washed with several changes of water to remove Cl ion and air-dried at room temperature. This was then heated at 110°C for 2 hours and stored for later use in a holder.

The paper chromatography was conducted by the

ascending procedure using a mixture of diisobutyl ketone, acetic acid, and water (40:25:5) (30).

Spots of amino nitrogen-containing phospholipids were made visible by the use of ninhydrin reagent and after that spots of choline-containing phospholipids were developed with aqueous phosphomolybdic acid solution, followed by reduction with acid SnCl₂ to molybdenum blue (31).

RESULTS

Results of Inositol Assay—Inositol contents of red cells (18) and spleen were usually very small, and the results of its assay are shown in Table I.

TABLE I
Inositol Content in Phospholipids

	Inositol (%)	P(%)	Inositol/P
Lipids of sheep red cells	0.22	1.38	0.027
" bovine " "	0.25	1.97	0.022
" horse " "	0.00	1.27	0
" human " "	{ 0.20 0.24	1.60	{ 0.021 0.026
Ether-sol. phospholipids of horse spleen	0.29	2.40	0.021
Ether-sol. phospholipids of bovine spleen	0.19	2.73	0.012

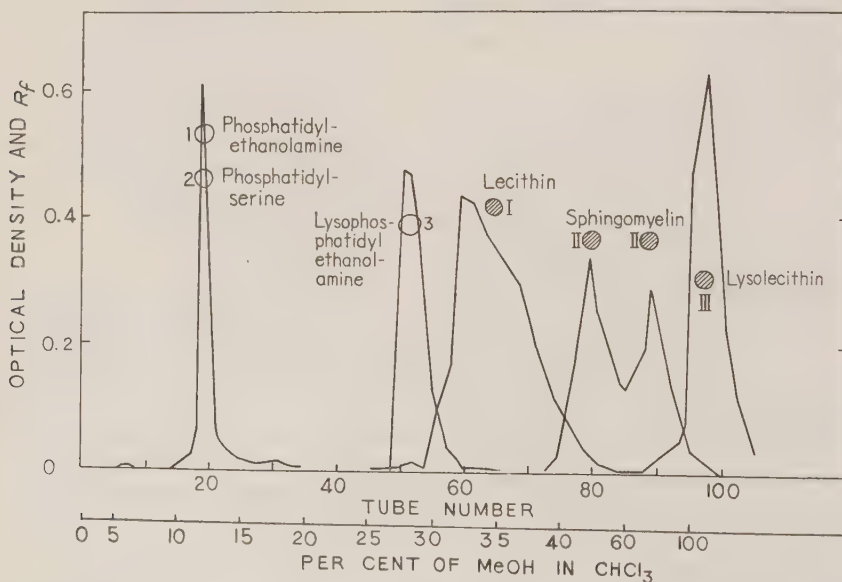


FIG. 1. Chromatography of authentic phospholipid samples on silica-paper and on silicic acid column.

Chromatography of Phospholipids

Paper Chromatography of Phospholipids on Silica-paper—Schematic paper chromatograms of each of the authentic samples* of phospholipids are shown in Fig. 1. Spot 1, 2, and 3 are phosphatidylethanolamine, phosphatidylserine, and lysophosphatidylethanolamine, respectively, all of them giving positive ninhydrin reaction. Spots I, II, and III are lecithin, sphingomyelin, and lysolecithin, respectively, which are stained blue on treatment with phosphomolybdic acid followed by reduction of their spots (31).

When phosphatidylethanolamine from egg yolk or ox brain cephalin, which had been stored in the air for a long time, was subjected to this paper chromatography, it gave two spots, one corresponding to that of original phosphatidylethanolamine and the other locating just below that of the phosphatidylserine. This unknown spot was later identified with lysophosphatidylethanolamine (33). Accordingly, it is reasonable that each of these peaks obtained by column chromatography contained a trace of lysophosphatidylethanolamine.

General Comments on Column Chromatography—Results of chromatography of authentic phospholipid samples on silicic acid column are shown in Fig. 1. Ninhydrin-positive phospholipids were eluted out by the solvent mixture of chloroform and decreasing amount of methanol (10–25 per cent methanol in chloroform). Bases of these fractions, usually ethanolamine, serine, and small amounts of other ninhydrin-positive materials, were detected, but their identification was not carried out.

* Authentic samples of phosphatidylethanolamine and phosphatidylserine were prepared by Folch's method (32). Phosphatidylethanolamine was also prepared from egg yolk lipids by chromatography on silicic acid column. Detailed procedure for the preparation of pure lysophosphatidylethanolamine will be described later in a paper of this series (33). Pure lecithin was prepared from egg yolk by the method of Rhodes *et al.* (34) and lysolecithin by Hanahan's procedure from egg lecithin (35). Sphingomyelin was obtained pure from blood stroma (1).

In general, the amount of ethanolamine was greater than that of serine (Tables II–X). A phospholipid eluted by chloroform containing 25 per cent of methanol was positive to ninhydrin and its spot was located just below that of phosphatidylserine on silicated paper chromatogram. This material showed hemolytic activity and was assumed to be lysophosphatidylethanolamine, as will be described later.

Phospholipids containing choline may generally be eluted by a solvent mixture containing higher concentration of methanol than 30 per cent. Generally, they were eluted in order of lecithin, sphingomyelin, and lysolecithin with increasing methanol concentration in the solvent mixture. Sphingomyelin was eluted out by the solvent mixture containing 40 to 60 per cent of methanol and separated into two peaks. However, these two peaks gave the same paper chromatogram. The similar observation caused by the change of the solvent composition was, more or less, observed on chromatography of other phospholipids.

Chromatography of Phospholipids from Stroma of Red Cells and Spleens—Results obtained by the chromatography of ether-soluble phospholipid mixtures from the stroma of man, cat, guinea pig, horse, rabbit, sheep, and cattle, together with those from the spleens of cattle and horse are shown in Figs. 2 to 10. Analytical results of the individual component in the main peaks are given in Tables II to X. The qualitative composition of phospholipids in these peaks are essentially similar to that already reported (12–17). Since majority of sphingomyelin together with glycolipids (ether-insoluble) had already been removed from phospholipid mixtures, these results gave only the patterns of phospholipids except sphingomyelin. Table XI gives the distribution of four glycerophosphatides, cephalin, lysocephalin, lecithin, and lysolecithin, calculated from the phosphorus content in each peak.

FIG. 2-10. Chromatography of ether-soluble phospholipids from mammalian red cells and spleens

— P, ---- Ninhydrin

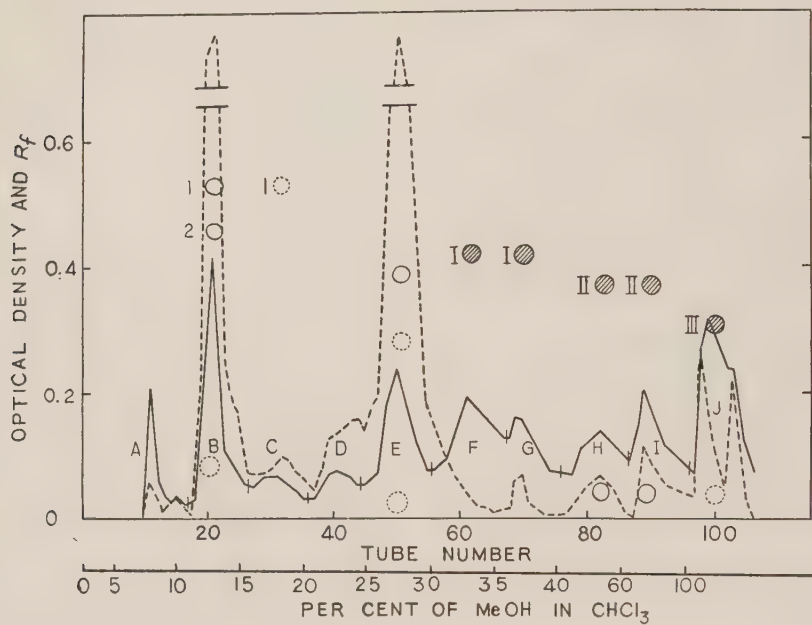


FIG. 2. Human red cells.

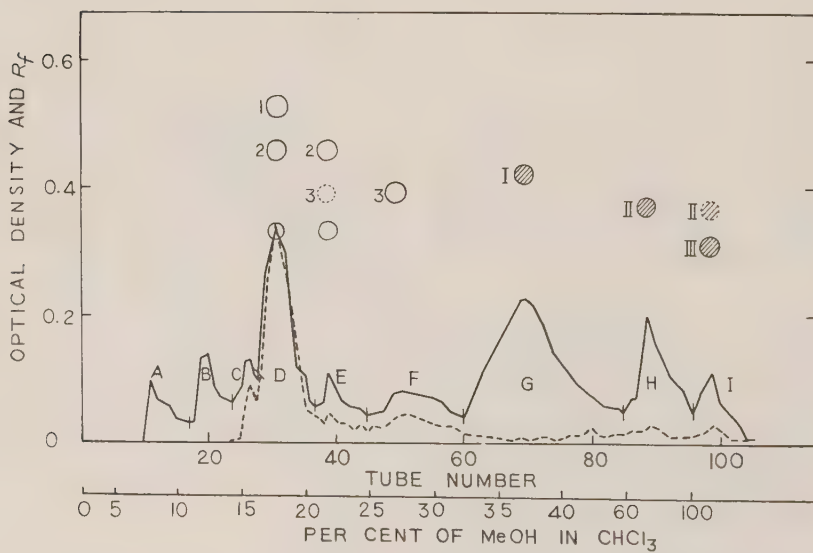


FIG. 3. Cat red cells.

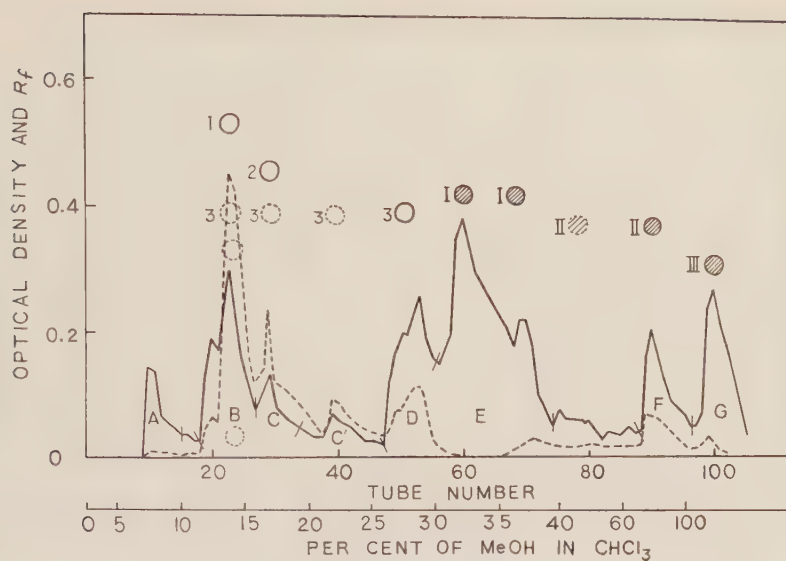


FIG. 4. Guinea-pig red cells.

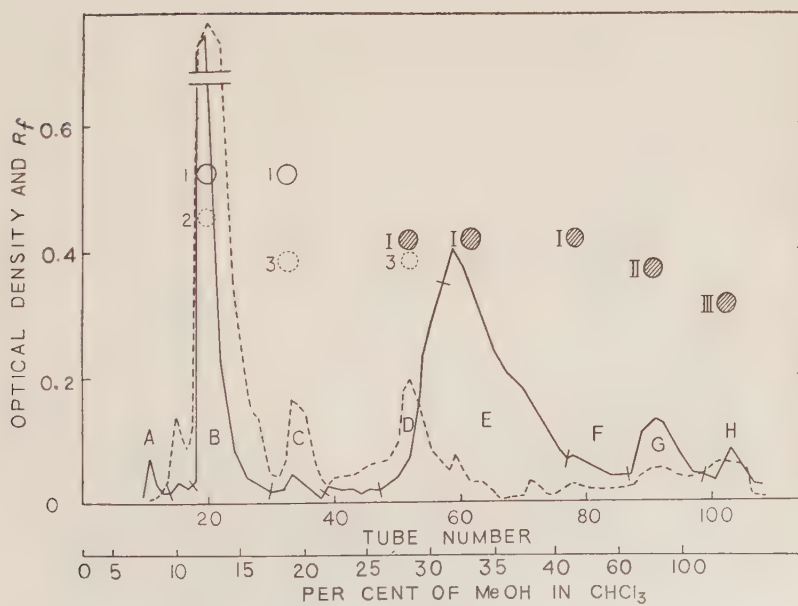


FIG. 5. Horse red cells.

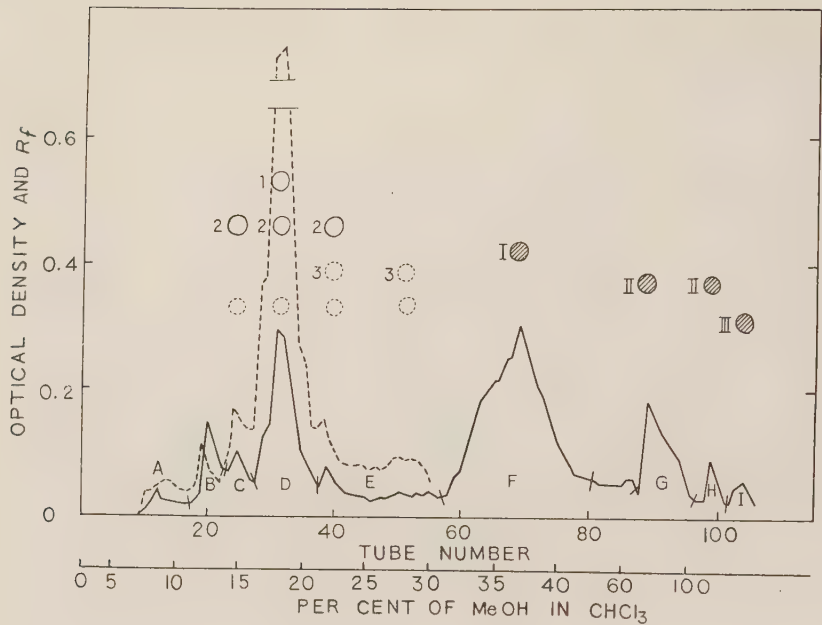


FIG. 6. Rabbit red cells.

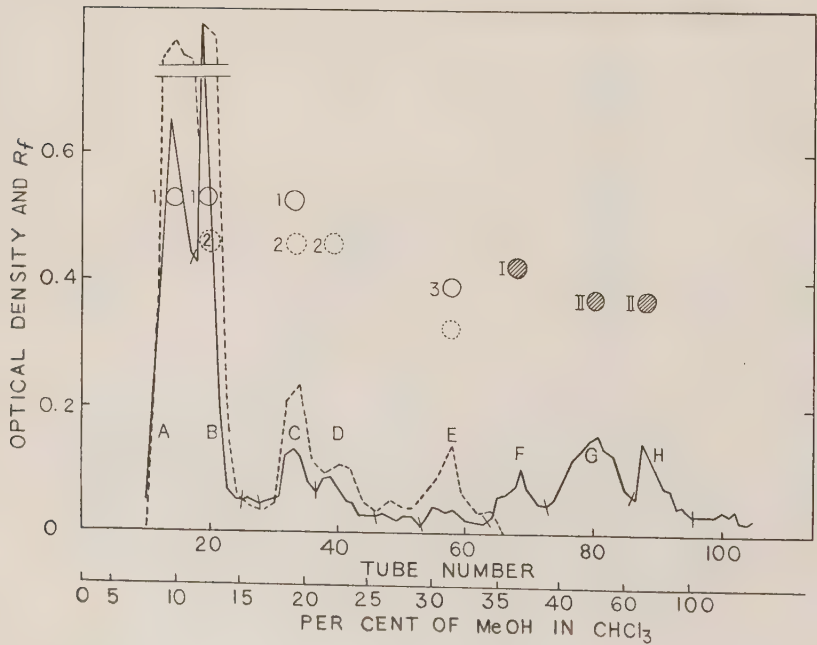


FIG. 7. Sheep red cells.

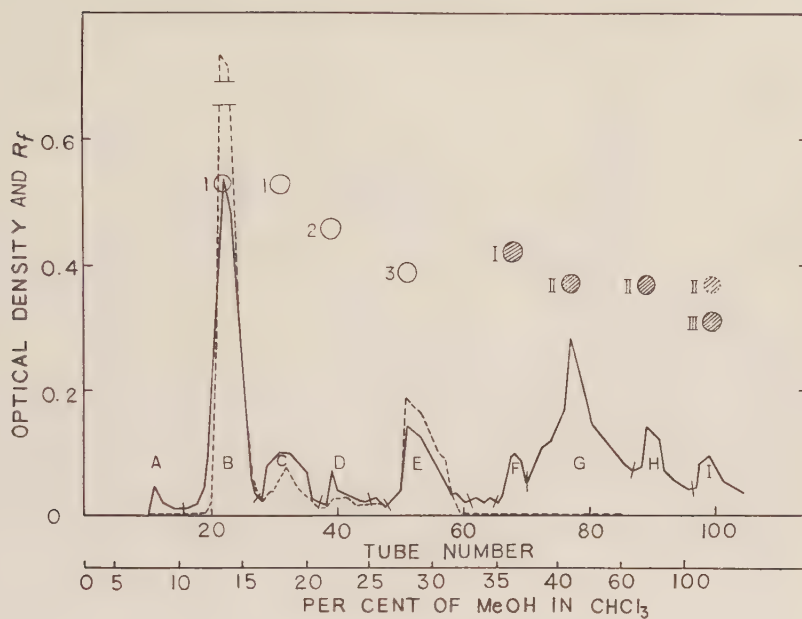


FIG. 8. Cattle red cells.

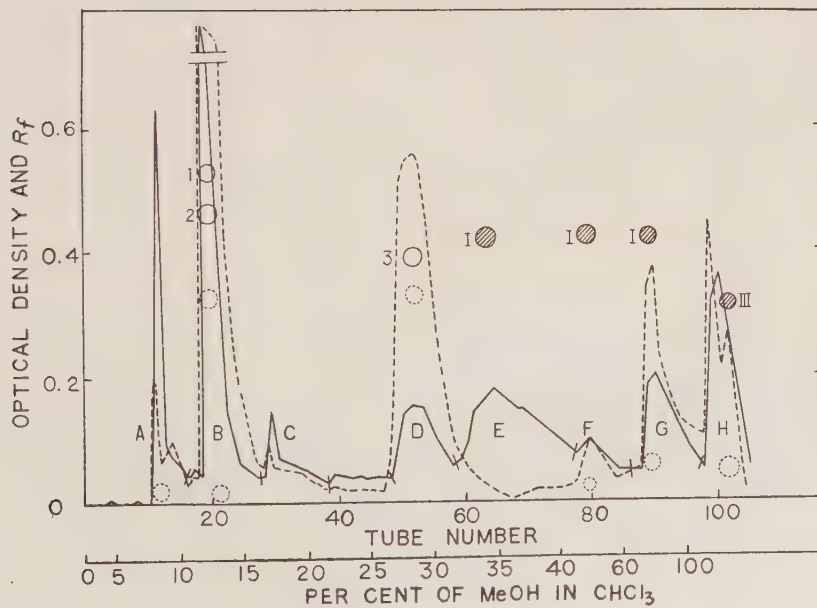


FIG. 9. Cattle spleen.

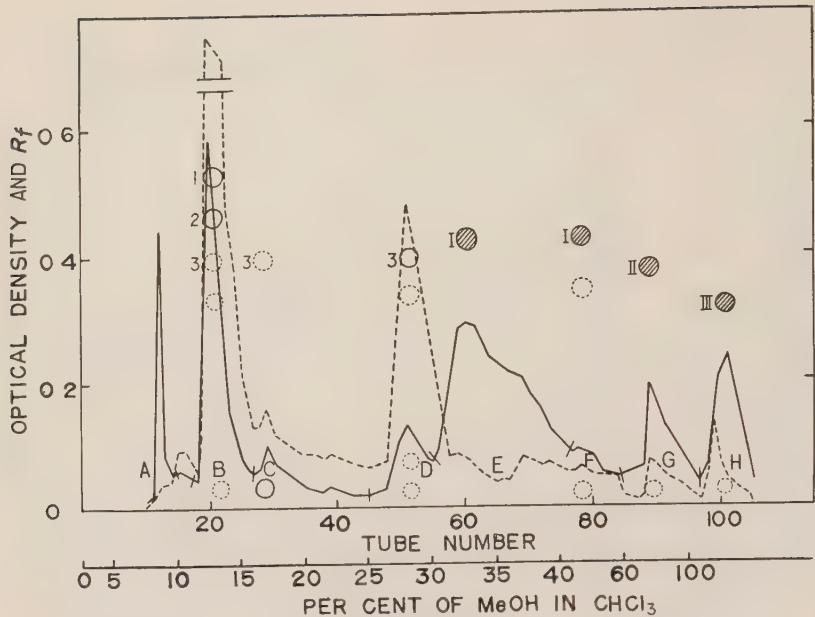


FIG. 10. Horse spleen.

TABLE II

Analysis of Human Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig.	1.40	3.19	0.97		8.82	2.96	1.01	12.77
E. Sol. Ph.	1.41	3.52	0.89	1.60		5.24	1.13	13.15
B	1.87	3.68	1.12	0.73		7.00	1.37	16.77
E	1.44	3.77	0.85	1.65	17.48			10.33
F	2.44	3.52	1.53		11.96			0.33
H	2.24	3.37	1.47		10.25			7.95
I	2.31	6.12	0.84		16.65			7.32

TABLE III

Analysis of Cat Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
B	0.91	3.72	0.54	1.32				
D	1.81	3.97	1.01	1.14		5.71	2.58	12.07
G	1.78	3.75	1.05	1.67	18.18			13.88
H	2.60	3.89	1.48		14.39			
I		4.05		1.10	21.62			16.12

TABLE IV

Analysis of Guinea-pig Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	2.21	3.76	1.30		21.0	2.06	1.48	
B	1.38	3.12	0.98	1.30		3.74	2.22	12.05
D	1.57	2.79	1.24	0.83	0	3.11	1.17	9.82
E	1.60	3.23	1.09	1.66	14.20			12.68
F	2.64	3.15	1.86		11.60			
G		4.36		0.94	10.08			

TABLE V

Analysis of Horse Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	1.83	4.00	1.01		14.36	2.41	0.68	10.88
B	1.71	3.82	0.99	1.54		6.40	2.23	13.49
D	1.78	3.48	1.13	1.36	12.24	1.84	0.67	12.97
E	1.80	3.48	1.14	1.85	14.50			10.89
G	2.84	3.90	1.61		14.35			
H	2.47	5.06	1.08	0.25	13.47			15.24

TABLE VI

Analysis of Rabbit Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	2.85	4.73	1.33		11.25	2.68	1.08	10.79
D	2.05	3.54	1.28	1.54		6.21	2.33	11.41
F	2.23	3.43	1.44	1.72	16.00			
G	2.92	3.80	1.70		15.18			

TABLE VII

Analysis of Sheep Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	1.81	4.03	0.99	0.41	1.22	5.2	1.01	
A	1.50	3.62	0.92	1.34	0	6.6	1.53	7.61
B	1.63	4.28	0.84	1.30	0	7.18	2.14	12.26
C	1.33	3.36	0.88	1.54	0	4.50	1.34	12.90
E		2.36		1.07	0	6.28	1.41	
F	1.47	3.34	0.97	1.44	15.29			10.70
H	2.76	4.10	1.47	0.35	15.87			

TABLE VIII

Analysis of Cattle Red Cell Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	3.13	4.40	1.57		1.73	6.90	1.95	11.35
B	1.98	3.84	1.14	1.45		7.47	1.09	13.62
E		5.43		0.89		11.0	1.54	

TABLE IX

Analysis of Cattle Spleen Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	1.67	2.73	1.35		6.05	3.22	1.00	10.73
A	1.49	1.98	1.66	2.14		1.84	0.76	8.91
B	1.97	3.29	1.32	1.50		4.86	3.32	11.37
D	2.47	4.10	1.33	0.78		6.40	3.13	13.72
E	1.51	3.67	0.91	1.42	12.34			12.11
G	3.42	3.74	2.02	1.49	10.10			12.44
H	2.99	3.54	1.37	1.04	16.22			12.62

TABLE X

Analysis of Horse Spleen Phospholipids Separated by Silicic Acid Column Chromatography

Fr.	N (%)	P (%)	N/P	E/P	Choline-Cl (%)	Ethanol-amine (%)	Serine (%)	Glycerol (%)
Orig. E. Sol. Ph.	1.47	2.40	1.35		8.40	3.22	0.85	9.39
A		3.46		1.29			0.80	14.65
B	1.94	2.90	1.48	1.75		4.62	1.56	11.44
D	2.45	4.75	1.14	0.84		6.00	1.52	13.31
E	1.67	3.44	1.07	1.70	13.45			13.23
G	3.14	3.53	1.97		10.53			
H	3.15	5.75	1.21	1.06	19.49			11.80

TABLE XI

Molar Percentages of Glycerophosphatides on Blood Stroma and Spleen

	Cephalin	Lysocephalin	Lecithin	Lysolecithin
Human stroma	29.0	17.5	30.5	23.5
Cat „	36.5	13.5	42.5	7.5
Guinea pig „	21.5	17.0	47.0	14.5
Horse „	33.5	2.5	60.5	3.5
Rabbit „	38.0	5.0	53.5	3.0
Sheep „	90.0	3.0	6.5	trace
Cattle „	63.0	18.5	7.0	11.0
Cattle spleen	30.0	11.0	43.0	15.5
Horse „	30.5	8.0	49.5	12.0

DISCUSSION

Separation of phospholipids from tissues of various animals was carried out effectively by the use of silicic acid column chromatography. The identification and determination of each phospholipid were made possible with the aid of chromatography on silica-impregnated paper. In order to avoid confusion, glycolipids together with sphingomyelin were removed from the phospholipid mixtures prior to the analysis. Therefore, the content of sphingomyelin was not determined.

The molar percentages of lecithin, cephalin, and their lyso-derivatives are listed in Table XI. Among different species, prominent differences which were thought to be genetically determined were observed in the phospholipid composition. Red cells of ruminants (cattle and sheep) were found to contain little or no lecithin as reported by Bürger *et al.* (15) and Turner *et al.* (16, 17). These differences may probably be responsible for different susceptibility of red cells of various animals to hemolytic action of snake venoms or *Cl. perfringens* toxin. Unexpectedly, a large amount of lyso derivatives was found in the stroma lipids of human, cat, guinea pig, and cattle red cells, but their biological significance has to be solved in future.

Since the role of spleen was thought to have some physiological correlation with the function of blood, constitutional relation of phospholipids was also expected between them. However, the results of chromatographic analyses showed no similarity in the phospholipid composition (except sphingomyelin) of spleen and red cells of the same species.

Inositol in red cells was found in every case to be a minor component, the amount of which was less than 0.3 per cent of that of total acetone-insoluble lipids, in good agreement with the result of Phillips *et al.* (18). In connection with the presence of phytic acid in avian erythrocytes (20) and metabolically active brain phosphoinositide (19), paucity of inositol in these anuclear, mature mammalian red cells might in some way be related with their metabolic inertness.

SUMMARY

1. Phospholipids from erythrocyte stroma of human, cat, guinea pig, horse, rabbit, sheep, and cattle, together with the spleens of cattle and horse, were separated by chromatography on silicic acid column and compared with each other.

2. The molar ratios of lecithin, cephalin, and their lyso derivatives were presented for each of the red cells and spleens. Results obtained by Bürger *et al.* and Turner *et al.* that red cells of ruminants contain little or no lecithin were also confirmed. Unexpectedly, a large amount of lyso derivatives was found in stroma lipids of human, cat, guinea pig, and cattle red cells.

3. Spleens and red cells of horse and cattle showed no similarity in their phospholipid compositions.

4. Inositol in red cells of sheep, cattle, horse, and human beings was found to be a minor component, the amount of which was in every case less than 0.3 per cent of that of total acetone-insoluble lipids. Ether-soluble phospholipids of horse and bovine spleens together with egg yolk contained an average 0.25 per cent of inositol.

The author wishes to acknowledge his great indebtedness to Prof. T. Yamakawa who made this work possible, and to Prof. T. Ukita for his constant encouragement. Thanks are also due to Miss Y. Natsume for her skilful assistance in technical work and to Miss M. Iwanaga for elementary analysis.

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Studies on Phospholipids

II. Phospholipase Activity of Clostridium Perfringens Toxin

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Alpha toxin of *Cl. perfringens* type A has been known to be responsible for hemolytic, necrotic, and lethal effects of the toxic bacterial filtrate (1). In relation to the mechanism of its hemolytic activity, the report of Macfarlane *et al.* in 1941 (2) is of interest in that the toxin hydrolyzed lecithin producing phosphorylcholine and diglyceride. Zamecnik *et al.*, in their detailed studies (3) on the phospholipase activity of this toxin, further indicated that sphingomyelin, phosphatidylethanolamine, phosphatidylserine, lysolecithin, cerebroside, and glycerylphosphorylcholine were not attacked by the toxin and that phosphatidylserine inhibited the phospholipase activity of the toxin by combining with calcium ion which was employed as activator of the reaction. However, Macfarlane later found that the toxin could hydrolyze sphingomyelin when a greater amount of toxin was employed and the incubation was prolonged (4). The phospholipase activity has been demonstrated also in the culture filtrate of a number of other clostridia, *viz.* *oedematiens*, *sordellii*, *chauvoei*, *sporogenes*, *centrosporogenes*, *tertium*, and *bifermentans*, but immunological property of the enzyme has been found different among those bacterial species. Furthermore, differences in hemolytic activity were also found among the toxins of these species (5, 6). For example, Oakley *et al.* (7) pointed out that alpha-toxin of *Cl. perfringens* readily hemolyzed sheep erythrocytes which were relatively insensitive to *Cl. oedematiens* toxin, but hardly affected horse erythrocytes which were very sensitive to *Cl. oedematiens* toxin. The report of Macfarlane (8) is worthy

of note in this connection that hemolysis of horse and sheep erythrocytes by these toxins was always preceded by decomposition of some of the phospholipids of the cells, and that the rate of hydrolysis was different between horse and sheep cells.

In the study of the composition of erythrocyte stroma phospholipids from various animals (9), the present author reported that sheep and bovine cells contain no or little lecithin, and consequently sphingomyelin is the sole choline-containing phospholipid in these cells, whereas horse, human, and rabbit cells contain a considerably large amount of lecithin and less amount of cephalin. Such differences in lipid composition seem to have bearings on the different rates of hydrolysis and hemolysis cited above.

The present study was undertaken to clarify chemical change of phospholipids in red cells, especially those of sheep, when they were subjected to the action of alpha toxin. It was thereby found that phosphatidylethanolamine, which had been considered to be resistant to the action of the toxin, was hydrolyzed by *Cl. perfringens* toxin, liberating phosphorylethanolamine.

MATERIALS AND METHODS

Phospholipids—Preparation of phospholipid mixture soluble in ether from red cell stroma was described previously (9). Pure phosphatidylethanolamine was prepared from egg yolk phospholipid mixture by silicic acid column chromatography (10, 11), followed by dialysis in a rubber membrane against petroleum ether (12). The dialyzed content was then evaporated to dryness and used as the authentic phosphatidylethanol-

amine (ester/P=2). Another set of samples of phosphatidylethanolamine and phosphatidylserine was prepared from ox brain by the method of Folch-Pi (13). Sphingomyelin was obtained from ether-methanol extract of red cell stroma (14) (sheep and human) and purified by treatment with diluted alkali according to the method of Thannhauser *et al.* (15). On analysis, these sphingomyelins gave N/P=2, ester/P=0.

Acid-soluble Phosphorus Compounds—Phosphorylcholine and phosphorylethanolamine were synthesized by the method of Plimmer and Burch (16).

Preparation of Toxic Material from the Culture Filtrate of Cl. perfringens—A strain of *Cl. perfringens*, PB6K, was grown in peptone culture medium enriched with some vitamins, salts, and fructose (17). After incubation at 35°C, for about 7 hours, the culture fluid was filtered rapidly through a Seitz filter pad. One liter of the fresh culture filtrate thus obtained was saturated with ammonium sulfate. The scum of crude toxic material which rose to the surface was rapidly skimmed off and placed on top of a filter paper pile. The crude toxic material on the paper was then dried in a vacuum desiccator over P₂O₅ and NaOH. The amount of the product was 4.7 g. (3.84 E.U./mg.). It was almost free from either phosphorus or calcium.

Analytical Methods—Phosphorus content was estimated by Allen's method (18), amino nitrogen of intact phosphatide by the Ninhydrin method of Moore and Stein (19) modified by Lea and Rhodes (20), ester by ferric hydroxamate method (21), N by micro-Dumas method, and Cl by Pregl's micro-Carius method. The potency of the alpha toxin was assayed by turbidimetry on a solution of lecithovitellin as described by van Heyningen (22).

Reversed-phase column chromatography for fatty acid analysis was carried out essentially by the method of Howard *et al.* (23) and Yamakawa *et al.* (24).

Hemolysis of Red Cells by Toxic Preparation from the Culture Filtrate of Cl. perfringens—Freshly obtained packed sheep red cells were washed repeatedly with saline and suspended in 3.5 volumes of saline. To the cell suspension was added one volume of a solution of *Cl. perfringens* toxin in borate-CaCl₂ buffer (pH 7). This was incubated at 37°C for one hour with vigorous shaking and cooled. After dilution with an equal volume of saline, trace of insoluble material was removed by centrifugation at 3,000 r.p.m. for 15 minutes. The amount of the toxin used was 50 mg. per 100 ml. of packed red cell suspension.

Paper Chromatography of Acid-soluble Phosphorus Compounds—Phospholipid sample (about 10 mg.) emulsified in 0.001 M CaCl₂ (0.5 ml.) was incubated at 37°C

with *Cl. perfringens* toxin (1 mg.). Continuous hydrolysis was obtained by adjusting pH to 7 with alkali during incubation. After several hours the reaction was stopped by the addition of trichloroacetic acid to the final acid concentration of 2.5 per cent. Resulting precipitate was centrifuged and supernatant was freeze-dried. The dried residue was dissolved in a small amount of distilled water and subjected to ascending paper chromatography in 80 per cent phenol. Spots were made visible by the use of ninhydrin reagent (0.1 per cent in butanol saturated with water) and Hanes reagent for phosphorus (25) successively.

Assay System of Phospholipid Hydrolysis by Cl. perfringens toxin—Titrimetric method was employed for the assay of hydrolysis rate.

The reaction mixture in typical experiments consisted of the following: Emulsion of substrate (phospholipid) containing 18 μ moles of lipid P per ml. and an equal volume of toxin solution (1 mg./ml.), both in 0.001 M CaCl₂, pH of which were previously adjusted to 7 with alkali. The mixture of substrate and toxin was incubated at 37°C and 2.0 ml. was pipetted out at regular intervals. The sampled material, after addition of an equal volume (2.0 ml.) of 94 per cent ethanol to stop the reaction, was titrated with 0.01 N methanolic NaOH using 0.1 per cent Bromothymol Blue in ethanol as indicator to estimate the hydrolysis rate.

Preparation of Hydrolysis Products from Cephalin and Sphingomyelin—Hydrolysis of cephalin and sphingomyelin by the toxin was carried out on a large scale in order to isolate decomposition products of these enzyme reactions. Details of these experiments were described in the section of 'Results'.

RESULTS

Changes in Composition of Stroma Lipids of Sheep Red Cells Hemolyzed by Cl. perfringens Toxin—From hemolysate of sheep red cells prepared in a manner described in "Method" section, stroma was precipitated by the addition of acetic acid, washed with water, separated by centrifugation, and lyophilized. The resulting material was extracted repeatedly with ether-methanol (1 : 1) at room temperature. The extract thus obtained was precipitated with acetone after removal of solvent and fractionated into ether-soluble and -insoluble fractions. The latter was further separated into pyridine-soluble (glycolipids) and -insoluble (sphingomyelin) fractions. The lipid

TABLE I
Difference in Stroma Lipid Composition between Intact Sheep Red Cells and Those
Hemolysed by *Cl. perfringens* Toxin

Cells	Dry weight of stroma analyzed (g.)	Ether-sol. phospholipid (lipid P, mg.)	Pyridine-insol. phospholipids (Sphingomyelin) (g.)	Pyridine-sol. lipids (Glycolipid) (g.)
Intact I	100	284 (ethanol-amine, 5%)	9.0	0.54
II	"	217	4.47	0.65
III	"	224	6.92	0.99
Hemolyzed	100	40 (ethanol-amine, 4%)	0.46	0.59

mixture derived from intact cells was also fractionated in the same manner. The amounts of these three lipid fractions (ether-soluble, pyridine-insoluble, and -soluble) are shown in Table I. Ether-soluble phospholipids decreased markedly as well as pyridine-insoluble phospholipid fraction (sphingomyelin) by the toxin, but pyridine-soluble lipid fraction (glycolipids) seemed to be unaffected.

Paper Chromatography of Acid-soluble Phosphorus Compounds—Lecithin, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, lysolecithin, lysocephalin, and ether-soluble phospholipid mixtures of stroma from human, sheep, and horse red cells were tested in the same manner as described above. The results are illustrated in Fig. 1. Lecithin and sphingomyelin were hydrolyzed by the toxin and gave only one spot which had the same R_f value (0.91) as synthetic calcium phosphorylcholine chloride when acid-soluble portion was chromatographed on filter paper in 80 per cent phenol. This result coincides with those of Macfarlane *et al.* (2, 4) and others (3). However, cephalin, which had been considered to be unhydrolyzable (3, 4), also gave a spot (R_f 0.47) corresponding to that of synthetic phosphorylethanolamine (16). Ether-soluble phospholipid mixtures from red cells which generally contain these three phospholipids, after hydrolysis by the toxin, gave two spots corresponding to those of phosphorylcholine and phosphorylethanolamine.

No water-soluble (acid-soluble) phosphorus

was released from these phospholipids in the absence of either calcium ion or the toxin. It was also found that phosphatidylserine,

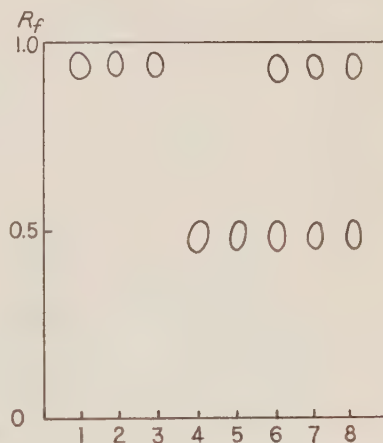


FIG. 1. Paper chromatography of acid-soluble phosphorus compounds.

Acid-soluble phosphorus compounds from:

2. lecithin, 3. sphingomyelin, 5. phosphatidylethanolamine, 6. ether-soluble phospholipids of sheep red cells, 7. ether-soluble phospholipids of horse red cells, 8. ether-soluble phospholipids of human red cells, 1. Ca-phosphorylcholine chloride, 4. phosphorylethanolamine. Lipid samples were incubated with *Cl. perfringens* toxin, then precipitated with trichloroacetic acid, and resulting supernatant was chromatographed on filter paper using 80% phenol as a developing solvent.

Phosphatidylserine, lysolecithin, and lysocephalin were also tested, but no acid-soluble phosphorus compounds were released from them.

lysolecithin and lysocephalin were not decomposed by the toxin.

Periodical Change of Phospholipid Hydrolysis by the Toxin—The hydrolysis rate was compared by the titrimetric assay method among lecithin, cephalin (sheep stroma), sphingomyelin, and ether-soluble phospholipid mixtures of sheep, cattle, horse, and human stroma. The results obtained are shown in Fig. 2.

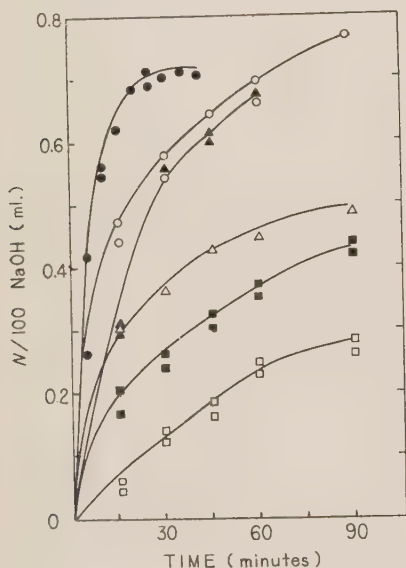


FIG. 2. Time course of phospholipid hydrolysis by *Cl. perfringens* toxin.

● lecithin ○ sphingomyelin ▲ ether-soluble human red cell phospholipids △ ether-soluble horse red cell phospholipids ■ ether-soluble sheep red cell phospholipids □ cephalin from sheep red cells phospholipids

Final concentration: Substrate, 9 μ mole/ml., toxin, 0.5 mg./ml. CaCl_2 , 0.001 M. Incubation was carried out at 37°C.

Lecithin was the most rapidly decomposed of the lipids. However, sphingomyelin was also hydrolyzed considerably rapidly and the result differing from that reported by Macfarlane (4). Cephalin, which is a mixture of phosphatidylethanolamine and phosphatidylserine, was hydrolyzed more slowly. Phospholipid mixtures obtained from sheep and bovine red cell stroma, containing less amount of lecithin (9), were decomposed rather slowly than those of human and horse red cells rich in lecithin

content (9). It was confirmed that no significant reaction occurred in the absence of calcium ion.

In Fig. 3 the results obtained by estimation of the actual amount of acid-soluble phosphorus released from phospholipids are shown.

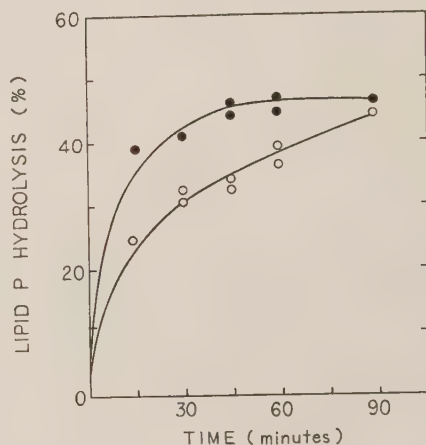


FIG. 3. Hydrolysis of phospholipid by *Cl. perfringens*.

● sphingomyelin
○ sheep stroma ether-soluble phospholipids

Final concentration: Substrate, 9 μ mole/ml., toxin, 0.5 mg./ml., CaCl_2 , 0.001 M. After incubation at 37°C, hydrolysis was determined by estimation of trichloroacetic acid-soluble P-compounds.

Effect of Substrate Concentration on the Hydrolysis—When phospholipid emulsion, 6 to 36 μ moles per ml. (final), was incubated with a certain amount of *Cl. perfringens* toxin at 37°C, for 30 minutes increase in substrate concentration caused a larger alkali consumption in titration, which indicated a greater liberation of acidic materials from the substrate by the toxin. However, in the case of lecithin, maximum hydrolysis was observed at concentration of 24 μ moles per ml. These results are shown in Fig. 4.

Effect of Toxin Concentration on the Hydrolysis—With a low concentration of the toxin (0.25–1.0 mg. per ml.) the extent of hydrolysis of phospholipids was nearly proportional to the toxin concentration when they were incubated at 37°C for 30 minutes. The results obtained

are illustrated in Fig. 5.

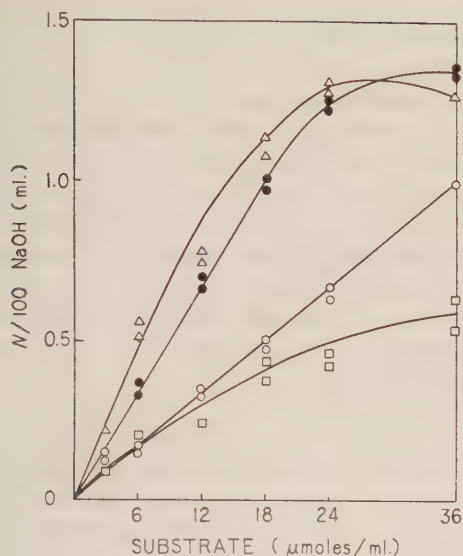


FIG. 4. Effect of substrate concentration on hydrolysis of phospholipids with *Cl. perfringens* toxin. ● Sphingomyelin ○ Sheep stroma ether-soluble Fr. △ Lecithin □ Sheep cephalin

Final concentration of toxin: 0.5 mg./ml.
Incubation: at 37°C for 30 min.

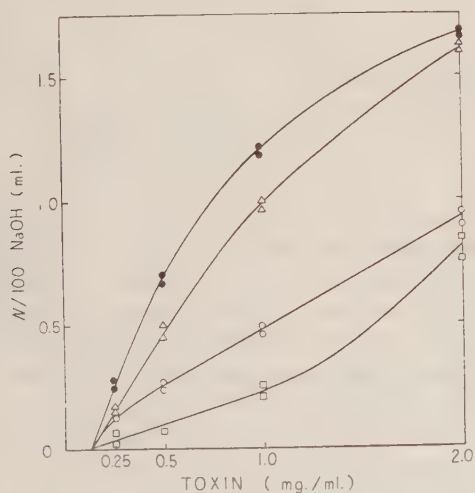


FIG. 5. Effects of toxin concentration on hydrolysis.

● Lecithin △ Sphingomyelin
○ Ether-soluble phospholipids of sheep stroma
□ Cephalin from sheep stroma

Final concentration: Substrate, 9 μmoles/ml.
CaCl₂, 0.001 M.

The reaction mixture was incubated at 37°C for 30 min.

Effect of NaF on the Enzyme Reaction—When NaF was added to the enzyme reaction medium in an equimolar amount of that of CaCl₂ present in the medium and incubated with toxin, no alkali was consumed on titration, indicating that NaF inhibited the enzyme reaction.

Hydrolysis of Cephalin by Cl. perfringens Toxin: Separation and Identification of Hydrolysates—Hydrolysis of ether-soluble phospholipids of sheep erythrocyte stroma (P, ca. 20 mg.) was carried out with 5 mg. of toxin. The pH of the reaction mixture was occasionally adjusted to 7 during the incubation. Three hours later, further 5 mg. of the toxin was added and incubation was continued. After about ten hours, the reaction mixture was cooled and extracted four times with ether by shaking and centrifugation. The resulting pooled ether layer and aqueous layer showed phosphorus content of 11.18 mg. and 9.39 mg., respectively. To the aqueous layer was added trichloroacetic acid to remove toxin and residual lipids, and resulting precipitate was removed by centrifugation. To the supernatant, after neutralization with KOH to phenolphthalein a few drops of 10% barium acetate solution and four volumes of absolute ethanol were added with precaution to prevent rise of temperature. Fine white precipitate thus formed was centrifuged down after cooling for 10 hours at 0°C and barium was removed by mixing with Amberlite IR-120 (H form) and a small amount of water. After repeating centrifugation and washing with a small amount of water, the supernatants were combined, concentrated *in vacuo* to one ml., and added with twenty volumes of absolute ethanol. On standing, fine crystals consisting of long prisms appeared, which amounted to 25 mg. It melted at 231°C under decomposition and showed no depression of the melting point on admixture with an authentic sample. Analytical values of this substance corresponded with these of phosphorylethanolamine.

Calcd. for C ₂ H ₅ O ₄ NP:	C 17.03, H 5.73,
	N 9.93, P 22.0
Found	: C 17.36, H 5.49,
	N 10.17, P 22.05.

Its chromatographic behavior on filter paper, melting point, and infrareds pectrum agreed well with these of the authentic specimen of phosphorylethanolamine.

Phosphorylcholine liberated from sphingomyelin, which was contained in the original ether-soluble phospholipid mixture, was found in the ethanolic supernatant after removal of the precipitate of barium phosphorylethanolamine and identified by paper chromatography.

Separation of diglyceride in a pure form from the mixture of ether-soluble phospholipids of sheep red cells failed, since it contained phospholipids other than cephalin, and furthermore the resulting diglyceride portion might be secondarily degraded by lipase action involved in the toxin. Hence the second trial of hydrolysis with chromatographically pure cephalin of egg yolk was undertaken.

Previous to the hydrolysis, cephalin was dialyzed in a rubber tube against petroleum ether for two days with several changes of the solvent. By means of this treatment, cephalin became free from dialysable materials such as sterols, glycerides, or fatty acids. Cephalin thus obtained was hydrolyzed by the toxin. After hydrolysis, 0.93 mg. of acid-soluble P was recovered, a result showing that 36 per cent of the original phosphorus (2.6 mg.) was hydrolysed. Ether extract of the reaction mixture was dialyzed in a rubber tube against petroleum ether and the dialyzate was evaporated to dryness in a water-bath under reduced pressure. The residue weighed 19 mg., corresponding nearly to the amount of diglycerides calculated from the rate of hydrolysis. It was found to contain glycerol when subjected to acrolein test, and ester linkages in 2.07 μ moles and free acids in 1.23 μ moles per mg. If the original egg yolk phosphatidylethanolamine contains fatty acids corresponding to an average C_{17} acid (26), it requires ester linkages of 3.31 μ moles per mg. Therefore, these findings indicate that diglyceride released from egg yolk cephalin by *Cl. perfringens* toxin might have undergone further degradation with liberation of free fatty acids by the action of lipase contained in the toxin

preparation. It may be assumed from this result that about 75 per cent of the diglycerides was changed to monoglycerides by the lipase action.

Action of Cl. perfringens Toxin on Tween 80—

In connection with the lipase activity described above, Tween 80 (0.5 per cent solution in water) was found to be hydrolyzed on incubation with the toxin at 37°C producing acidic material which consume alkali on titration (Fig. 6). This finding strongly suggests the presence of lipase which splits the ester linkage of Tween 80 (sorbitan monooleate polyoxyethylene).

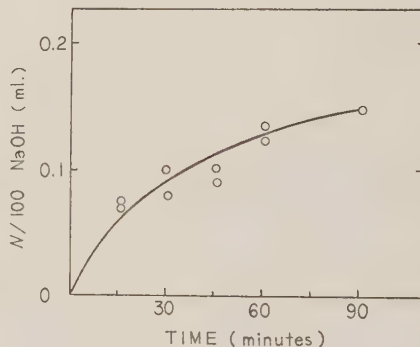


FIG. 6. Hydrolysis of Tween 80 by *Cl. perfringens* toxin.

Final concentration: Substrate, 0.5% (v/v)
Toxin, 0.5 mg./ml.
 $CaCl_2$, 0.001 M.

Hydrolysis of Sphingomyelin by Cl. perfringens Toxin: Separation and Identification of Hydrolyzates—Two and the eight tenth g. of sphingomyelin obtained from human erythrocyte stroma was emulsified with 200 ml. of 0.02 M $CaCl_2$. To the emulsion, after adjustment to pH 7 with alkali, about 300 mg. of the toxin was added in three portions at intervals of 6 hours. The mixture was incubated at 37°C, with occasional adjustment of pH to 7 with 0.1 N NaOH, for about 20 hours. At the end of incubation 72.5 per cent of phosphorus became soluble in water.

The aqueous portion (I) was separated from insoluble materials (II) by filtration, concentrated to about 30 ml., and, after dialysis in a cellophane bag against distilled

water with several changes of water, the combined dialyzate was lyophilized. The residue thus obtained was extracted twice with hot absolute ethanol and filtered. Then the combined filtrate was concentrated to 32 ml. and added with 8 ml. of 0.5M CaCl_2 . The resulting white precipitate was centrifuged, washed successively with 60 and 80 per cent ethanol, and dried over P_2O_5 in an evacuated desiccator. On analysis it corresponded to calcium phosphorylcholine chloride.

Calcd. for $\text{C}_5\text{H}_{13}\text{O}_4\text{NPClCa} \cdot 4\text{H}_2\text{O}$: C 18.21, H 6.42, N 4.25, P 9.46, Cl 10.75.

Found: C 18.72, H 6.14, N 4.30, P 9.25, Cl 10.40.

The water-insoluble portion (II) was extracted with hot acetone and filtered while hot through a warmed funnel. This process was repeated three times. When the pooled filtrate was allowed to cool, white crystalline material precipitated, melting at around 85°C . This substance weighed 1.655 g. and contained 0.13 per cent of phosphorus. It was then subjected to column chromatography with a column (22 mm. in diameter) of florisil (50 g.) in chloroform. The sample dissolved in 20 ml. of chloroform was loaded on to the column, washed with 180 ml. of the same solvent, and the column was eluted with 400 ml. of chloroform containing 5 per cent of methanol. The eluate obtained from this chloroform left a white residue after removal of the solvent. On recrystallization from acetone the residue gave a white crystalline material (III) melting at $84\text{--}87^\circ\text{C}$. It contained no phosphorus. Its analytical values (C 78.06, H 12.45, N 2.41%) almost corresponded to those of ceramide with fatty acid of $\text{C}_{20}\text{--}\text{C}_{22}$ and its infrared spectrum also corresponded to that of authentic ceramide reported by Marinetti *et al.* (27), but it was thought that these data were not sufficient to conclude that this material to be pure ceramide. Fatty acid residue combined to nitrogen atom of ceramide obtained from sphingomyelin by the toxin was considered to be a mixture of acids differing in length of their carbon chain. Hence this material was subjected to

further degradation with hydrochloric acid.

Degradation of III by 5 N HCl—A mixture of 300 mg. of the material with 60 ml. of methanolic hydrochloric acid (methanol:conc. HCl =1:1, v/v) was refluxed on a boiling water bath for 10 hours. The reaction mixture was concentrated *in vacuo*, extracted three times with ether, and after washing with water the ether extract was dried over anhydrous sodium sulfate. The aqueous washings were combined with the mother liquor. White waxy material (103 mg.) remained on evaporation of ether from this extract.

The material (fatty acid esters) was saponified with alkali as usual and subjected to reversed-phase column chromatography (23, 24). As shown in Fig. 7, the fatty acid frag-

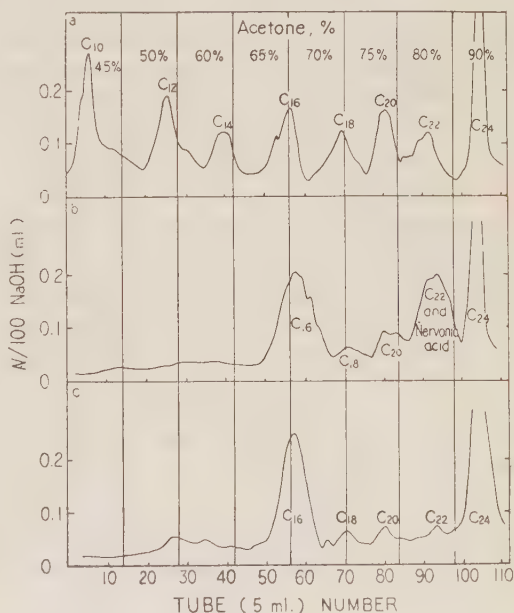


FIG. 7. Reversed-phase column chromatography of fatty acids.

- Standard
- Fatty acids from ceramide
- Reduced fatty acids from ceramide

ment was found to be a mixture of palmitic, stearic, arachidic, nervonic and especially a large amount of lignoceric acid. This gives the explanation for the previous finding that the ceramide obtained gave analytical results

corresponding to that with fatty acid of C_{20} — C_{22} as its components. The fatty acids recovered from the ceramide corresponded to those of original sphingomyelin (24), thus indicating that the enzymatic cleavage proceeds irrespective of the fatty acid composition of original sphingomyelin.

The aqueous portion was made alkaline with NaOH and extracted with ether. This extract was dried over anhydrous sodium sulfate after washing with water. Brown oily material, obtained on evaporation of the solvent and weighing 115mg., was dissolved in acetone and a small amount of insoluble material was centrifuged off. To the resulting supernatant giving positive ninhydrin reaction sulfuric acid in absolute ethanol was added to pH 4, the precipitate that formed was centrifuged, washed repeatedly with absolute ethanol, and finally dried over P_2O_5 *in vacuo*. On analysis of nitrogen, this product corresponded to sphingosine sulfate or a relating substance.

Calcd. for $(C_{18}H_{37}O_2N)_2 \cdot H_2SO_4$: N	4.02
Found	: N 4.14.

By this degradation study, it was concluded that III was a mixture of ceramide with mainly palmitic, nervonic, and lignoceric acids bound to its nitrogen atom.

DISCUSSION

The results presented herein indicated that phosphatidylethanolamine, although it had been considered to be resistant to the action of the toxin, was hydrolyzed by the toxin of *Cl. perfringens* producing phosphoryl-ethanolamine. In view of the enzyme specificity and of metabolic significance of phospholipids, this finding is thought to be very interesting.

Evidence was obtained suggesting that diglycerides, which were produced from phosphatidylethanolamine by this phospholipase-C action, was further hydrolyzed with lipase contained in the toxin preparation to produce free fatty acids and monoglycerides. Such lipase activity has been known to be present in the toxin of *Cl. oedematiens* type A, but

not well established previously in the case of *Cl. perfringens*. This lipase activity was further confirmed by the hydrolytic effect of the toxin on ester linkage of Tween 80 (Fig. 6).

Phosphorylcholine produced from sphingomyelin by *Cl. perfringens* toxin has been identified (4). However, identification of ceramide, which is supposed to be produced as well in the reaction, has not yet been successful. In the present study, production of ceramide in the reaction was definitely confirmed by its isolation and characterization of its chemical structure.

Lecithin was highest in the rate of hydrolysis by *Cl. perfringens* toxin, followed by sphingomyelin, and cephalin was much lower than the other two phospholipids. The maximum hydrolysis of lecithin was attained at the substrate concentration of $24 \mu\text{moles per ml.}$ In the case sphingomyelin and cephalin, higher concentrations seemed necessary to obtain the maximum hydrolysis. These results are inconsistent with those of Macfarlane and others (2, 3, 4), and suggest the possibility that the toxin preparation of *Cl. perfringens* may contain different enzymes for lecithin, sphingomyelin, and cephalin, despite Macfarlane's opinion (4, 5, 6, 8). This possibility seems to be further supported by the findings of Macfarlane that gamma-toxin of *Cl. oedematiens* could hydrolyze phospholipids of horse red cells (composed mainly of lecithin, sphingomyelin, and cephalin*) as rapidly as beta-toxin of this clostridium and alpha-toxin of *Cl. perfringens*, but hardly decomposed phospholipids of sheep red cells (composed mainly of cephalin and sphingomyelin*), which was decomposed by either alpha- or beta-toxin, in spite of using the same amount in 'lecithinase' activity. This observation may lead to an assumption that alpha (or beta)-toxin may contain an enzyme different from 'lecithinase', which can hydrolyze certain phospholipids such as phosphatidylethanolamine but not lecithin, and is not present in gamma-toxin. To reach any conclusion, purification of the responsible

* Phospholipid composition of these red cells was described in the previous paper of this series (9).

enzymes is of primary importance.

SUMMARY

1. Enzyme activity of decomposing phosphatidylethanolamine as well as lecithin and sphingomyelin was demonstrated in a culture filtrate of *Cl. perfringens* type A, PB6K.

2. Hydrolysis rate of phospholipid by the toxin was highest in lecithin, followed by sphingomyelin, and was lowest in phosphatidylethanolamine.

3. Phosphorylethanolamine produced from phosphatidylethanolamine by the toxin, together with phosphorylcholine and ceramide from sphingomyelin, were separated and identified.

4. Evidence has been obtained suggesting the presence of lipase which hydrolyzes glycerides and Tween 80 liberating fatty acids.

The author expresses his thanks to Prof. T. Yamakawa for his kind advices, to Prof. T. Ukita, for his continuous encouragements and interests in this work, and to Dr. R. Murata and Miss T. Yamada for their co-operation in microbiological work. He also wishes to thank Miss Y. Natsume for her capable assistance and Miss M. Iwanaga for analytical work.

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Studies on Phospholipids

III. Enzymatic Formation of Lysophosphatidylethanolamine

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Snake venoms are known to contain an enzyme designated as phospholipase-A which splits off one fatty acid residue from lecithin to produce lysolecithin which has cytolytic activity upon various cells, especially on red cells (1, 2, 3, 4, 5, 6, 7). Hanahan found that the enzymatic reaction proceeds in etheral solution, and examined its kinetics and chemical nature of the products (8). It has been said that cephalin is also hydrolyzed to lysophosphatide by phospholipase-A from snake venom or pancreatic preparation (2, 4, 6, 7, 9), but the production of lysocephalin from cephalin by the action of these enzyme preparations has not yet been confirmed by its isolation and chemical identification. Chargaff (6) reported that lysocephalin was produced together with lysolecithin when egg yolk was incubated with snake venom in phosphate buffer, but no lysocephalin was detectable when cephalin, alone or with lecithin added, was treated with snake venom. Fairbairn claimed that evidence was obtained for the ability of snake venom to produce lysocephalin together with lysolecithin from phospholipid mixtures isolated from beef brain (7). Long *et al.* (9) presented evidence for lysocephalin formation from etheral solution of cephalin in the presence of ammonia and calcium ion, but they did not isolate it. Chemical preparation of lysocephalin and its chemical and biological properties were reported by Debuch (10).

The present communication describes the formation of lysocephalin (lysophosphatidylethanolamine) from egg yolk lipids by

the action of a snake venom, its purification, and examination of its chemical and hemolytic properties.

MATERIALS AND METHODS

Enzyme—Freeze-dried snake venom from *Trimeresurus flavovirides* (Hallowell), a species of *Crotalidae*, was used as phospholipase-A.

Substrate—Egg yolk emulsion and its lipid mixture prepared by essentially the same method as described by Lea *et al.* (11) were used.

Chemical Assay—Total phosphorus was determined by Allen's method (12), ethanolamine and serine by fluorodinitrobenzene method (13), ester by ferric hydroxamate method (14), and glycerol by Blix' method (15).

Infrared Spectra—Hitachi infrared automatic recording spectrometer was used (KBr pellet).

Chromatography—Silicic acid column chromatography was carried out in a manner described previously (16).

Paper chromatography of phospholipids was conducted on silica-paper by Marinetti's procedure (17).

Reversed-phase column chromatography for analysis of fatty acids was carried out by the modified method of Howard *et al.* (18).

Assay of Hemolysis—Fresh sheep red cells were repeatedly washed with saline and suspended in a phosphate buffered saline (0.85 per cent saline 3: M/15 phosphate buffer (pH 7) 1, v/v) to make 0.5% cell suspension. One ml. of the suspension and 0.5 ml. of lysophosphatide solution in the same diluent were mixed and incubated at 37°C for 30 minutes. The observation was made after cooling the solution.

RESULTS

Enzyme Reaction and Extraction of Lipid—Yolk of five eggs was homogenized with 150

ml. of phosphate buffer ($M/15$, pH 7), and incubated at 37°C with 10 mg. of snake venom. After about fifteen hours, the reaction mixture was dialyzed against running water for three days to remove inorganic phosphates completely and the dialyzed material was freeze-dried. The dried material was then extracted repeatedly with a mixture of chloroform and methanol (1:1), and the extracts were combined, from which solvents were removed under reduced pressure. Total phosphorus content of the residual material was 280 mg. The material contained ether-insoluble lysophospholipids and a small amount of ether-soluble phospholipids consisting of lecithin and cephalin.

Comparative Chromatographic Investigation of Phospholipids from Intact and Snake Venom-treated Egg Yolk—Chromatographic separation of phospholipids was conducted on silicic acid column. In Fig. 1a is shown the result of chromatography of intact egg yolk phospholipid mixture. Cephalin was eluted by chloroform containing 10 per cent of methanol and lecithin by chloroform containing 30–35 per cent methanol. A part of lecithin was eluted by chloroform containing 15 per cent of methanol. A small amount of lysolecithin was eluted by methanol. In Fig. 1b is illustrated the result of chromatography of snake venom-treated egg yolk phospholipid mixture. In contrast with intact egg yolk phospholipids (Fig. 1a), venom-treated egg yolk phospholipids were devoid of cephalin and lecithin (Fig. 1b), a fact indicating that snake venom had decomposed cephalin as well as lecithin. Cephalin was found to produce a ninhydrin-positive phosphatide, which was identified as lysocephalin.

Effect of Snake Venom on Isolated Phospholipids—Phospholipid mixture of egg yolk, composed mainly of lecithin and cephalin, was treated with snake venom in the same manner as in the case of whole egg yolk. Phospholipids were then extracted from the reaction mixture, dialyzed against water, and freeze-dried. The result of chromatography of the phospholipid mixture on silicic acid column is shown in Fig. 1c. A considerable

amount of lysolecithin with relatively small amount of lysocephalin were formed by the action of snake venom.

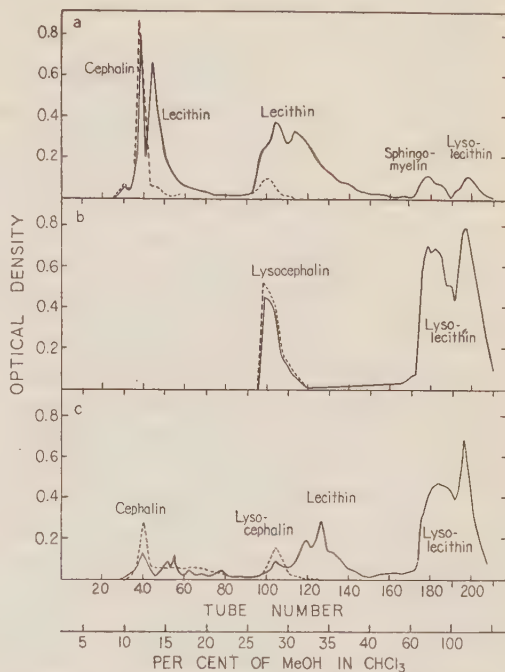


FIG. 1. Chromatography of phospholipids from intact and snake venom-treated egg yolk on silicic acid column.

- Phospholipids from intact egg yolk
 - Phospholipids from snake venom-treated egg yolk
 - Phospholipids after treatment with snake venom on isolated egg yolk lipids.
- P; -- Ninhydrin

Purification and Analysis of Lysocephalin (Lysocephalidylethanolamine)—Materials in the peak eluted by chloroform containing 25 per cent methanol (Fig. 1b) were collected and dialyzed in a cellophane tube against distilled water for two days. The dialyzed content gave only one ninhydrin-reactive spot when subjected to ascending chromatography on silica-paper in diisobutyl ketone-acetic acid-water mixture (40:25:5). Since the material, even after prolonged dialysis, was found to contain inorganic matters, it was then subjected to electro dialysis and freeze-dried. White powder thus obtained was

TABLE I
Analytical Properties of Lysocephalin

	C	H	N	P	N/P	Ester/P	Glycerol	Ethanolamine
Calcd. for :								
C ₂₁ H ₄₄ O ₇ NP ¹⁾	55.67	9.78	3.08	6.82	1	1	20.03	10.7
C ₂₃ H ₄₈ O ₇ NP ²⁾	57.36	10.05	2.91	6.43	1	1	19.12	9.78
C ₂₅ H ₅₂ O ₇ NP ³⁾	58.91	10.29	2.75	6.08	1	1	18.07	9.24
Found :	55.57	9.86	2.95	6.40	1.02	0.98	20.95	13.0
	55.82	9.51	3.11	6.36	1.08	0.97		

- 1) Lysophosphatidylethanolamine with palmitic acid
- 2) Lysophosphatidylethanolamine with stearic acid
- 3) Lysophosphatidylethanolamine with arachidic acid

dissolved in a warm mixture of chloroform and methanol (1:1, v/v) and filtered through a warmed funnel. When cooled, the filtrate gave a fine, colorless precipitate, which was collected by centrifugation. The material thus obtained was not so hygroscopic as lysolecithin and melted at 197°C under decomposition. It was insoluble in ether, chloroform, methanol, water, and pyridine, but slightly soluble in warm water and a mixture of chloroform and methanol. The analytical results of this material shown in Table I indicate that snake venom might remove one fatty acid residue from phosphatidylethanolamine, leaving its lyso derivative.

Mild Alkaline Hydrolysis of Lysocephalin—To the material (40 mg.) dissolved in a mixture of methanol (4 ml.) and carbon tetrachloride (4 ml.), 0.4 ml. of 2*N*-NaOH (50% methanolic) was added and heated at 40°C for 30 minutes. When cooled, it was treated with 4 ml. Amberlite IRC-50 (H form). After filtration, the resin was washed successively with 3 ml. portions of methanol-carbon tetrachloride, methanol, and water. The washings were combined with the filtrate and concentrated to about 3 ml. at 30°C under a reduced pressure. The condensate was extracted four times with ether after acidification with 0.1*N* HCl. From the ether extract, on evaporation of the solvent, 19 mg. of fatty acids (A) was obtained. Aqueous portion (B) was concentrated to dryness *in vacuo* at 30°C after neutralization with 0.1 per cent am-

monia. The residue was redissolved in 0.2 ml. of water and chromatographed on a filter paper (Toyo No. 53) using two solvent systems (80% phenol and *tert.* butanol-trichloro-

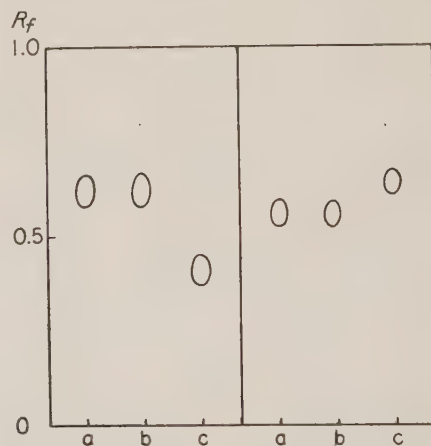


FIG. 2. Paper chromatography of water-soluble splitting product from lysocephalin by mild alkaline hydrolysis.

- a. Glycerophosphorylethanolamine
- b. Water-soluble hydrolysate from lysocephalin
- c. Phosphorylethanolamine

Chromatogram in the left was obtained by using 80% phenol, and that in the right using *tert.* butanol-trichloroacetic acid-water mixture (*tert.* butanol-water 62/38 v/v, trichloroacetic acid 10% w/v)

acetic acid-water mixture (19)) by the ascending procedure. Results obtained are shown in Fig. 2. These results suggested that glycerophosphorylethanolamine was produced

on alkaline hydrolysis of lysocephalin under mild condition.

This water-soluble hydrolysate (B) was further degraded with *N* HCl in a glass-stoppered test tube in a boiling water-bath for 40 minutes. After cooling, it was made alkaline with barium hydroxide solution, followed by addition of four volumes of absolute ethanol. The precipitate (I) formed on standing at 0°C was centrifuged. The supernatant was separated, freeze-dried, and redissolved in a very small amount of water. Trace of insoluble material which occurred on addition of absolute ethanol was removed by filtration. The filtrate was concentrated *in vacuo* (II). The precipitate (I) was suspended in a small amount of water and decationized with Amberlite IR 120 (H form). The aqueous acid solution thus obtained was concentrated *in vacuo* and subjected to paper chromatography in 80% phenol, which resulted in detection of glycerophosphoric acid (Fig. 3). From the

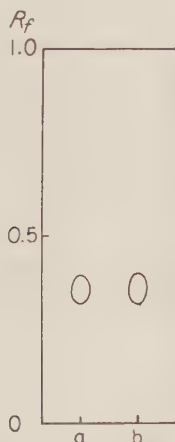


FIG. 3. Paper chromatography of phosphorus-containing hydrolysate from glycerophosphorylethanolamine (GPE) by hydrochloric acid.

- Glycerophosphoric acid
- P-containing hydrolysate of GPE from lysocephalin by HCl. Solvent system: 80% phenol.

supernatant (II) of barium precipitate (I), ethanolamine was identified on paper chromatograms both intact form and as a DNP-

derivative (Fig. 4). These findings show

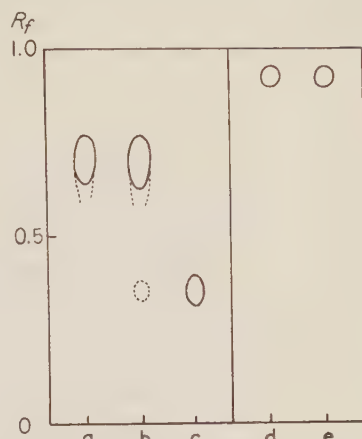


FIG. 4. Paper chromatography of basic hydrolysate from glycerophosphorylethanolamine (GPE) by hydrochloric acid.

- Ethanolamine
- Basic (phosphorus-free) hydrolysate of GPE from lysocephalin by HCl.
- Serine.
- d and e are 2,4-dinitrophenyl derivatives of a and b, respectively. Solvent system: 80% phenol, and butanol-ethanol-water (4: 1: 5, for DNP-derivatives)

that glycerophosphorylethanolamine produced by mild alkaline hydrolysis of lysocephalin was further degraded with HCl liberating glycerophosphoric acid and ethanolamine.

The fatty acid fragment (A) was analyzed by reversed-phase column chromatography (18). The results obtained are shown in Fig. 5. As shown in Fig. 5, the chromatographic pattern of fatty acids obtained from lysocephalin remained unchanged after catalytic reduction with PtO_2 , a fact indicating that they were saturated acids which consisted of palmitic and stearic acids.

Comparison of Infrared Spectra between Lyso-phosphatidylethanolamine and Lysolecithin—The infrared spectrum of lysophosphatidylethanolamine differed markedly from that of phosphatidylethanolamine, whereas the spectrum of lysolecithin bore a strong resemblance to that of lecithin, as will be seen from Fig. 6.

Hemolysis of Sheep Red Cells by Lysophosphatides—Hemolytic activity of lysophosphatidylethanolamine was compared with

that of lysolecithin. The results obtained are shown in Table II. Lysophosphatidylethanolamine showed stronger hemolytic effect on sheep red cells than lysolecithin. Hemolytic activity of this preparation of lysophosphatidylethanolamine was also higher than that of the chemical preparation from ox brain cephalin reported by Debuch (10).

TABLE II

Hemolysis of Sheep Red Cells by Lysophosphatides

Amount of lysophosphatide added to cell suspension. ($\mu\text{g./ml.}$)	100	50	25	12.5	6.25	3	1.5
Lysophosphatidylethanolamine	+	+	+	+	+	+	—
Lysolecithin	+	+	+	+	+	—	—

Assay of hemolysis was as follows: A mixture of 1 ml. of 0.5% sheep red cells and 0.5 ml. of lysophosphatide solution was incubated at 30°C for 30 minutes. After cooling, the degree of hemolysis was read.

DISCUSSION

Lysocephalin obtained by the phospholipase-A action of snake venom on egg yolk had one ester linkage per molecule. On hydrolysis under mild alkaline condition, the lysocephalin produced fatty acids and glycerophosphorylethanolamine, which was further degraded by hydrochloric acid to liberate glycerophosphoric acid and ethanolamine. Accordingly, a structure of monoacylglycerophosphorylethanolamine (lysophosphatidylethanolamine) is proposed for the lysocephalin.

Despite a number of studies on the action site of snake venom phospholipase-A on lecithin (or cephalin), it is still doubtful whether the enzyme splits the ester linkage of alpha or beta position in these phospholipids. In the present work, it seemed interesting to determine the site of the enzyme action on egg yolk cephalin, but it was not done since sufficient amount of the material was not available.

Lysophosphatidylethanolamine described herein is thought to be fundamentally similar in its structure to that reported by Debuch (10). However, the former is insoluble or

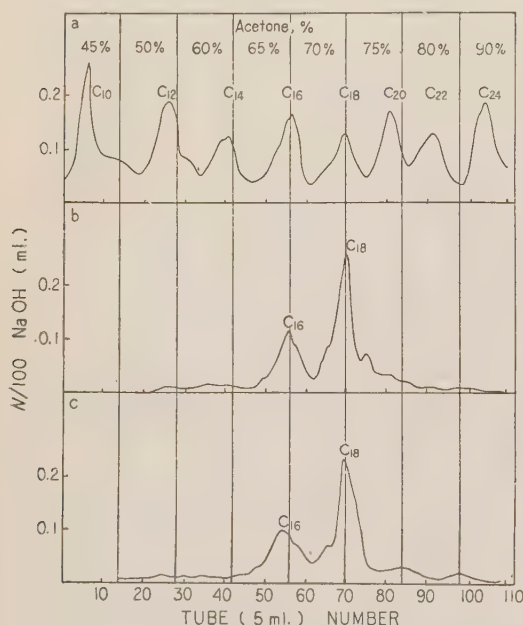


FIG. 5. Reversed-phase column chromatography of fatty acids from egg yolk lysocephalin.

- a. Standard
- b. Fatty acids from lysocephalin
- c. Reduced fatty acids from lysocephalin.

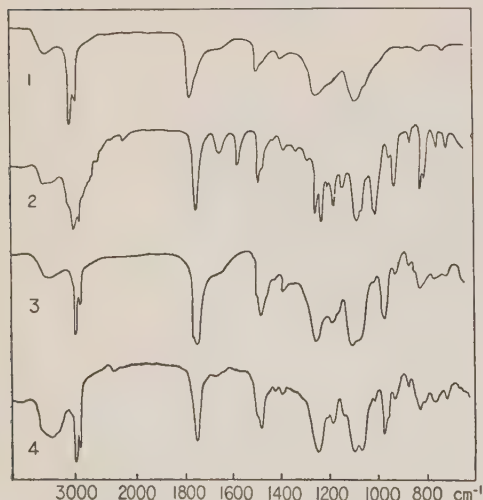


FIG. 6. Infrared spectra of cephalin (1), lysophosphatidylethanolamine (2), lecithin (3), and lysolecithin (4).

slightly soluble in ordinary solvents and not hygroscopic, whereas the latter had been reported to be soluble in hot pyridine as well as in warm water and hygroscopic. Optical rotation of the preparation could not be determined because of the lack of a suitable solvent. Since lysolecithin is soluble in chloroform and lysocephalin insoluble, the different solubilities should conveniently be used for their separation.

Although no definite assignment can now be made for the infrared absorption bands of lysophosphatidylethanolamine obtained in the present series of work, detailed analyses of these bands will be possible when a synthetic sample of this phospholipid become available.

SUMMARY

1. Formation of lysophosphatidylethanolamine from egg yolk phosphatidylethanolamine by the action of phospholipase-A of snake venom was confirmed by its isolation and characterization of its chemical structure.

2. Fatty acid composition of the lysophosphatidylethanolamine was shown to be a mixture of palmitic and stearic acids.

3. Hemolytic activity of lysophosphatidylethanolamine was compared with that of lysolecithin.

The author is very grateful to Prof. T. Yamakawa for his valuable advices. He also wishes to thank Prof. T. Ukita for his constant

encouragement and helpful suggestions. Thanks are also due to Miss Y. Natsume for her skilful technical assistance and to Miss M. Iwanaga for elementary analyses.

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The Bacterial Decomposition of Indoleacetic Acid*

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Kotake showed that the indole nucleus is decomposed enzymatically and kynurenine is formed from tryptophan. This was confirmed later by Knox (1) and by Hayashi (2). Ichihara and Sakamoto (3) also showed that indole was decomposed by an indole-splitting bacterium which was isolated from air to CO₂, H₂O and NH₃ via isatin and anthranilic acid.

The decomposition of indoleacetic acid by peroxidase has been studied in detail, but little is known about its degradation products.

We have isolated a microorganism from air, and studied the metabolic pathway by which indoleacetic acid is decomposed by this microorganism.

EXPERIMENTALS

1. Oxygen uptake was determined with a Warburg manometer.

One ml. of cell suspension (about 10 mg. dry weight) and 2.0 ml. of 1/15 *M* phosphate buffer, pH 7.2, were placed in the main chamber of the Warburg vessel. The central well contained 0.1 ml. of 50% KOH. Ten moles of indoleacetic acid in *M*/15 phosphate buffer, pH 7.2, was placed in the side arm. The total volume was 3.0 ml.

2. Fluorescent materials were determined by a Photovolt fluorometer using filter B HG-1 and N-440.

After incubation, the reaction mixture was acidified with sulfuric acid, and 8 ml. of ether was added. The fluorescent materials were extracted into the ether, and the fluorescence in ethereal layer measured.

3. Paper chromatography was carried out one-dimensionally using *iso*-propanol, NH₄OH, water (8:1:1) as solvent.

4. Column chromatography—A 0.8×50 cm. column of Amberlite IR 112 H⁺ form (200-300 mesh) was used. The column was eluted with a mixture of *M*/10 citrate buffer, pH 4.5 and ethanol (4:1).

RESULTS

The bacterium used was isolated from air. After adaptation to indoleacetic acid, it degraded the compound oxidatively. The oxygen uptake was inhibited by the addition of α,α' -dipyridyl. However the violet fluorescence in the reaction mixture during indoleacetic acid decomposition increased after addition of α,α' -dipyridyl to the system (Fig. 1 and Table I). The fluorescence was studied by paper and column chromatography. This compound had an *R_f* value of 0.56 on the paper chromatograms using the above solvent. This spot coincided with the spot of authentic anthranilic acid (Fig. 2). Another spot of *R_f* 0.85 was also observed. The incubation mixture was also chromatographed on an Amberlite IR 112 column as described above. One and a half ml. fractions were collected in a fraction collector. Indoleacetic acid was not eluted from the column with the eluent mentioned above. The fluorescent material which was eluted from the column was extracted into ether. The ether was evaporated and the residue was dissolved in distilled water. This solution was shown to have the same absorption spectrum as authentic anthranilic acid (Figs. 3 and 4), and gave a positive Tsuda reaction. Anthranilic acid was not formed from indoleacetic acid under anaerobic conditions (Table II). Bacteria adapted to indoleacetic acid were unable to decompose other indole derivatives as shown in Fig. 5. The decomposi-

* This work was presented at the 5th Kinki Local Meeting of the Japanese Biochemical Society at Kyoto, in November, 1958.

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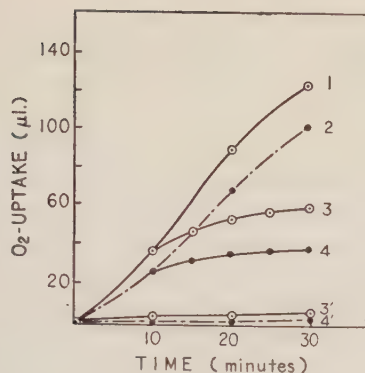


FIG. 1. Decomposition of indoleacetic acid and anthranilic acid by bacteria adapted to indoleacetic acid.

1. Indoleacetic acid
2. Anthranilic acid
3. Indoleacetic acid was incubated with bacteria for 10 min., and then 0.25 ml. of α, α' -dipyridyl was added.

3'. Bacteria were preincubated with α, α' -dipyridyl for 10 min., and then indoleacetic acid was added.

4. Anthranilic acid was incubated with bacteria for 10 min., and then 0.25 ml. of α, α' -dipyridyl was added.

4'. Bacteria were preincubated with 0.25 ml. of α, α' -dipyridyl for 10 min., and then anthranilic acid was added.

TABLE I

Appearance of a Fluorescent Material during Indoleacetic Acid Decomposition

Substrate	Addition	Fluorescence
Indoleacetic acid	—	3200
"	0.1 ml. α, α' -dipyridyl	13000
"	0.25 ml. "	18000
"	0.5 ml. "	15000
"	1.0 ml. "	10000

Experimental conditions were same as in Fig. 1 except that incubations were carried out in test tubes. α, α' -Dipyridyl was added 10 min. after indoleacetic acid was incubated with bacteria. Incubation for 30 min. at 37°C.

tion of the anthranilic acid formed from indoleacetic acid by this organism was completely inhibited by α, α' -dipyridyl.

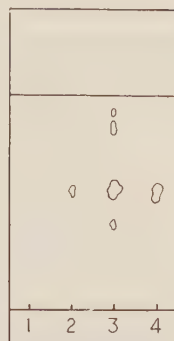


FIG. 2. Paper chromatography of an incubation mixture.

1. Bacteria only
2. Bacteria plus indoleacetic acid
3. 0.25 ml. of α, α' -dipyridyl was added 10 min. after indoleacetic acid was incubated with bacteria

4. Reference anthranilic acid
Incubation for 30 min. at 37°C. Solvent: Isopropanol, NH_4OH , water (8:1:1).

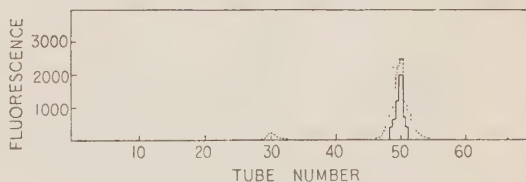


FIG. 3. Column chromatography of an incubation mixture.

Solid line, reference anthranilic acid
Dotted line, fluorescent material in the incubation mixture.

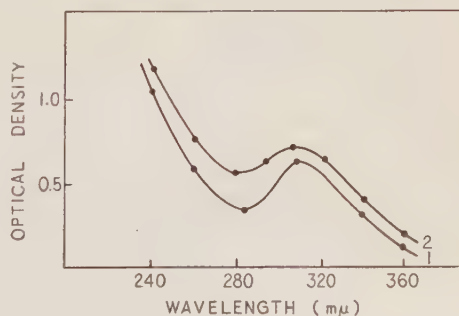


FIG. 4. Absorption spectrum of the fluorescent material in a reaction mixture.

1. Reference anthranilic acid
2. Fluorescent material in a reaction mixture

In order to elucidate the mechanism of

TABLE II
Formation of Anthranilic Acid under
Anaerobic Condition

Condition	Addition	Fluorescence
Aerobic		700
Anaerobic		160
Aerobic	0.25 ml. α, α' -dipyridyl	7500
Anaerobic	,,	310

Experimental conditions were same as in Fig. 1.
Incubation for 20 min. at 37°C.

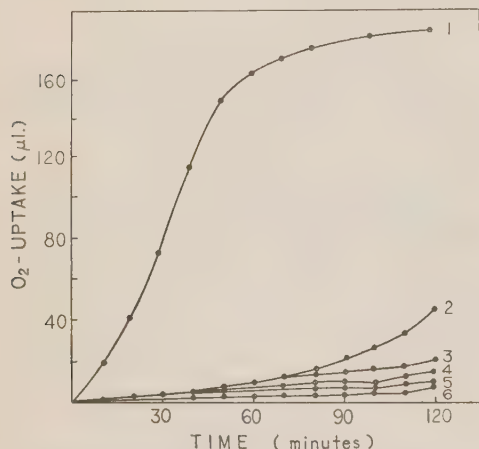


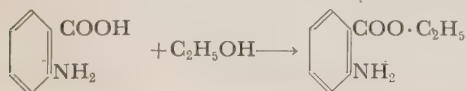
Fig. 5. Decomposition of various indole derivatives by indoleacetic acid-adapted bacterio.

10 μ moles of each substrate were used. Experimental conditions were same as in Fig. 1.

1. Indoleacetic acid
2. Indole
3. Indolepropionic acid
4. Indolebutyric acid
5. Indolecarboxylic acid
6. Scatole

the splitting of the indole ring, *o*-formaminobenzoylacetic acid, a possible intermediate, was synthesized.

Synthesis of the Ethyl Ester of o-Formaminobenzoylacetic Acid: *Step 1*—Thirty two g. of anthranilic acid and 200 ml. of ethanol saturated with HCl were mixed and heated at 90–100°. After 24 hours, the mixture was



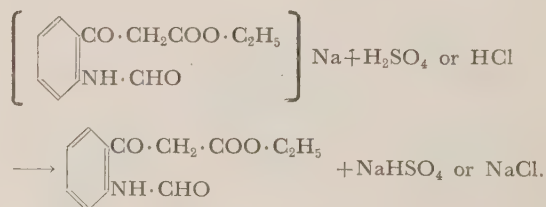
mixed with a solution of NaHCO_3 (70 g./l.), and the oily ethyl ester of anthranilic acid was obtained. The ester was extracted with 300 ml. of ether, mixed with 3 g. of Na_2SO_4 and allowed to stand overnight. The ether was evaporated after removal of the Na_2SO_4 , and about 31 g. of the ethyl ester of anthranilic acid were obtained.

Step 2—Formylation. Twenty ml. of 90 % formic acid were added to the ethyl ester, and the mixture boiled gently for 30 minutes. After cooling, the mixture was concentrated under reduced pressure for 30 minutes at 80°C.



Distillation of the concentrate at 135°–142°C gave about 29 g. of *o*-formaminobenzoylacetic acid ethyl ester. About 18.3 g. of *o*-formaminobenzoylacetic acid ethyl ester and 7.5 g. of $(\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5)\text{Na}$ were mixed and heated for 2 to 3 hours at 130°–140°C on an oil bath. Removal of the $\text{CH}_3\text{COOC}_2\text{H}_5$ under reduced pressure resulted in a loss of weight of about 4 g.

One hundred ml. of ether were then added at 70°C.



Step 3—Six ml. of concentrated HCl, 40

ml. of water and $\left[\text{C}_6\text{H}_4(\text{NH}\cdot\text{CHO})\text{CO}\cdot\text{CH}_2\text{COO}\cdot\text{C}_2\text{H}_5 \right] \text{Na}$

were mixed, well shaken, and the resulting NaCl in the aqueous layer was removed.

The crystals of $\text{C}_6\text{H}_4(\text{NH}\cdot\text{CHO})\text{CO}\cdot\text{CH}_2\cdot\text{COO}\cdot\text{C}_2\text{H}_5$

were soluble in ether.

o-Formaminobenzoylacetic acid synthesized had the following properties:

2,4-dinitrophenylhydrazine reaction	+
Tsuda reaction	—
Diazo reaction	—
Folin reaction	—
Fluorescence	+

The crystals were dissolved in ether and chromatographed on paper, and the fluorescent portion was eluted with ether. The ether was evaporated and the compound recrystallized from water. About 900 mg. of crystalline material were obtained.

The ethyl group of *o*-formaminobenzoyl acetic acid ethyl ester was split in acid, but the formyl group was also removed under these conditions. The amino group condensed with the carbonyl group to form a quinoline ring. Therefore, the ethyl ester was used as substrate. Fig. 6 shows the decomposition of

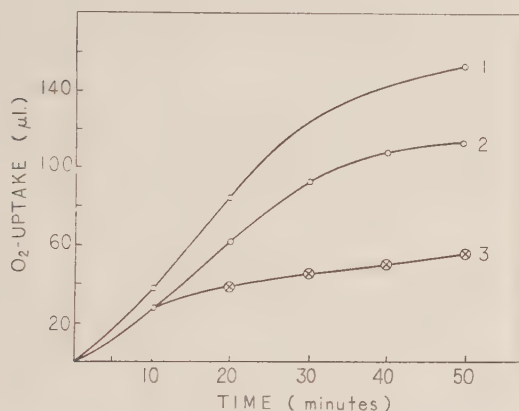


FIG. 6. Decomposition of synthetic *o*-formaminobenzoylacetic acid ethyl ester by indoleacetic acid-adapted bacteria

1. Indoleacetic acid
2. *o*-Formaminobenzoylacetic acid ethyl ester
3. *o*-Formaminobenzoylacetic acid ethyl ester was incubated with bacteria for 10 min., and then 0.25 ml. of α, α' -dipyridyl was added.

ethyl *o*-formaminobenzoylacetic by indoleacetic acid adapted bacteria. Addition of α, α' -dipyridyl had no effect on the decomposition of *o*-formaminobenzoylacetic acid. Ethanol was also decomposed by this bacterium, but much more oxygen was consumed in the degradation of *o*-formaminobenzoylacetic acid ethyl ester than with ethanol.

A paper chromatogram of the reaction mixture is shown in Fig. 7 demonstrating the

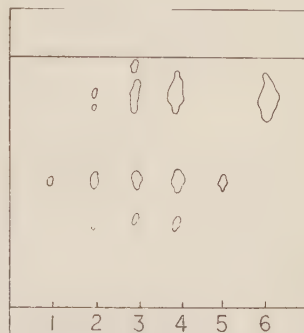


FIG. 7. Paper chromatography of an incubation mixture

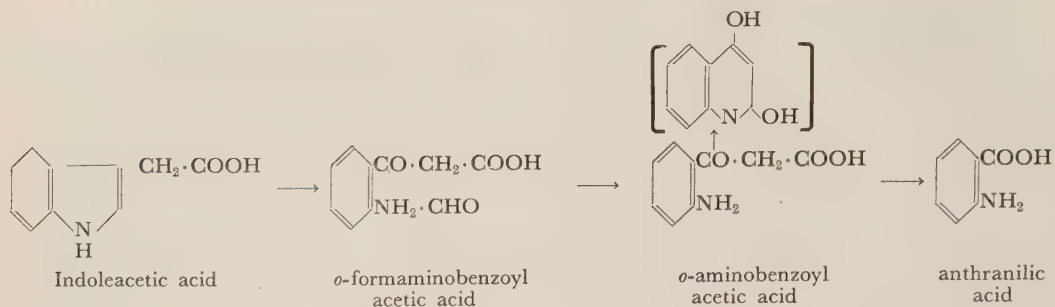
1. Indoleacetic acid
2. 0.25 ml. of α, α' -dipyridyl was added 10 min. after indoleacetic acid was incubated with bacteria
3. *o*-Formaminobenzoyl acetic acid ethyl ester
4. 0.25 ml. α, α' -dipyridyl was added 10 min. after *o*-formaminobenzoyl acetic acid ethyl ester was incubated with bacteria
5. Reference anthranilic acid
6. Reference *o*-formaminobenzoyl acid ethyl ester

formation of anthranilic acid. An extract of rabbit liver also converted *o*-formaminobenzoylacetic acid to anthranilic acid.

The fluorescent material which was formed during indoleacetic acid decomposition, having an R_f value of 0.85, might have been *o*-formaminobenzoylacetic acid. However, its identity could not be established since free *o*-formaminobenzoylacetic acid could not be synthesized. Attempts to obtain cell free extracts of the enzyme which decomposes indoleacetic acid by grinding, freezing and thawing, sonic oscillation, lysozyme, and acetone treatment were unsuccessful. Only dried cells had the ability to decompose indoleacetic acid.

DISCUSSION

It is concluded from the above experiments that indoleacetic acid is decomposed as follows:



Knox and Mehler isolated formylkynurenine as an intermediate in the conversion of tryptophan to kynurenine. We have also shown in this study that splitting of the indole ring occurs between the α and β carbons. Enzymatic studies on the mechanism of the split were impossible because a cell-free extract could not be obtained.

o-Formaminobenzoilacetic acid also but not indoleacetic acid was decomposed by liver extract. This may indicate that animals lack the enzyme which cleaves the indole ring of the latter.

SUMMARY

1. The bacterium used, after adaptation

to indoleacetic acid decomposed it oxidatively. *o*-Formaminobenzoylacetic acid was an intermediate in the conversion of indoleacetic acid to anthranilic acid.

2. The ethyl ester of *o*-formaminobenzoylacetic acid was synthesized.

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Studies on the Amphoteric Properties of Taka-amylase A

II. Unmasking of Acid Binding Sites*

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Hydrogen ion titration curves of proteins provide information concerning the number and dissociation constants of various kinds of ionizable groups that are accessible to titration. In addition, when some ionizable groups form intramolecular bonds, their presence can sometimes be detected from their abnormal ionization. The authors have previously reported that about twenty of the phenolic hydroxyl groups in Taka-amylase A (*Asp. oryzae* α -amylase, TAA) ionize slowly and irreversibly above pH 10.5 concomitantly with the alkali-denaturation of TAA. They inferred that these groups are participating in the maintenance of the intact structure of TAA through the formation of intramolecular bonds (1). The pH titration study was designed to get further information on the ionization of other groups in TAA in the hopes of finding what kind of groups forms intramolecular bonds with the phenolic hydroxyl groups.

The present paper deals with studies on the titration curves of TAA in acid pH region. An increase in the number of acid binding sites was observed below pH 4.3 and was ascribed to the unmasking of carboxylate groups which were incorporated in the native protein structure perhaps through the formation of intramolecular hydrogen-bonds.

EXPERIMENTAL

Taka-amylase A was prepared from "Takadiastase Sankyo" and recrystallized three times according to the method of Akabori *et al.* (2). A stock solu-

tion of TAA free from dialyzable calcium ions was prepared as described in the previous paper (1). The concentration of TAA was estimated spectrophotometrically with a Cary spectrophotometer, model 14 MP. The extinction of TAA in an acetate buffer (pH 5.6; $\mu=0.1$) was assumed to be $E_{1\text{cm}}^{1\%}=22.1$ at 2785 Å. The molecular weight of TAA was taken as 53,000, as determined from the terminal nitrogen (3) and light scattering**.

Standard 1.00 *N* hydrochloric acid and 1.00 *N* potassium hydroxide for volumetric analysis were purchased from Wako Pure Chemical Industries, Ltd. and were standardized against sodium carbonate and potassium acid phthalate. Potassium chloride was of special grade and was used without further purification. Distilled water deionized by passage through a mixed ion-exchange column was used throughout.

Measurement of pH was made by a Beckman pH meter, model GS, using the A scale. Beckman potassium acid phthalate buffer (4.00 at 20°) was used as a standard buffer.

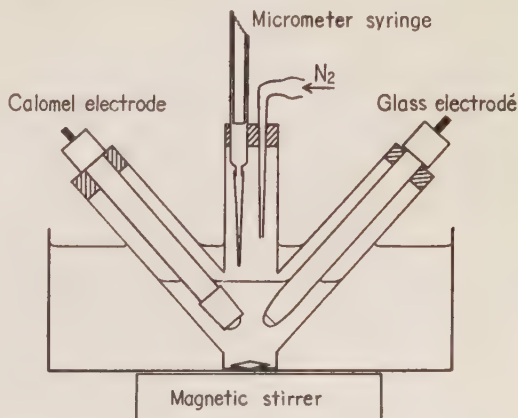


FIG. 1. Titration cell.

* Presented at the 13th Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1960.

** K. Hamaguchi, Unpublished data.

The titration cell used was designed by the authors and is shown in Fig. 1. This apparatus can be used either for the continuous or the point-by-point titration method. Twenty ml. of TAA solution (0.5–1.5%) was required to obtain a titration curve. A Beckman No. 40498 all purpose glass electrode and a No. 40451 sleeve type calomel reference electrode were used. Acid or alkali (usually 1.00 *N*) was delivered from an Agla micrometer syringe with stirring. Purified nitrogen was streamed through the cell to exclude carbon dioxide. The cell was thermostatically regulated by dipping it in a water-bath at constant temperature. After each titration, the pH meter was standardized. If the meter had an error of more than 0.02 pH unit, the titration was repeated. All the data were obtained at $20 \pm 0.1^\circ$ and ionic strength of 0.1. In order to calculate titration curves from the raw data (pH against the ml. of acid or alkali added to the solution), it was necessary to know the relation between the pH and the molarity of free hydrogen ion in the solution (m_{H^+}). This was found by determining an apparent activity coefficient γ' from the titration of pure solvent, which is defined as

$$pH = -\log m_{H^+} \gamma' \quad \text{Eq. (1)}$$

In pH-stat experiments pH values were controlled manipulatively to 0.02 pH unit by addition of 0.1 *N* hydrochloric acid from the micrometer syringe, with stirring.

Optical rotation was measured at $20 \pm 0.5^\circ$ with a Hitachi photoelectric spectropolarimeter. To avoid formation of a precipitate in acid pH region, no electrolyte was added except the acid required to adjust the pH and the potassium chloride in the stock solution of TAA (ionic strength, 0.01–0.04).

RESULTS

Isoionic Point of TAA and pH Shift of Isoionic Solution—For the analysis of the titration data, the isoionic point of TAA was measured before the titration experiments. The simplest method for the determination of the isoionic point is to measure the pH of a protein solution passed through an ion-exchange column, as described by Dintzis (4). It was not expected that this procedure could be applied to TAA, since its solubility is very low around the pH value expected as the isoionic point. It was found, however, that about a 0.5% solution of TAA could be passed successfully through a Dintzis column. No apparent precipitation occurred in the ion exchange

column (about 1 minute was required for the solution to pass through the column), nor in the effluent during the time required to collect enough solution to measure the pH value. It was found that the pH value of the isoionic TAA solution shifted slowly to the alkaline side. Fig. 2 shows the shift of pH in the solution eluted from the column in the presence and absence of 0.1 *M* potassium chloride. By extrapolation of the pH against time curve to time zero the isoionic point of TAA was judged to be about 3.79 at 20° either in the presence or in the absence of potassium chloride. Precipitation began after about 5 minutes in the presence of potassium chloride and after about 45 minutes in its absence. Therefore the observed pH shift is not primarily due to the formation of a precipitate.

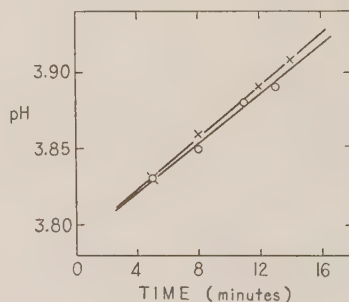


Fig. 2. Spontaneous pH shift of an isoionic solution of TAA in the presence (—○—) and in the absence (—×—) of 0.1 *M* KCl at 20° .

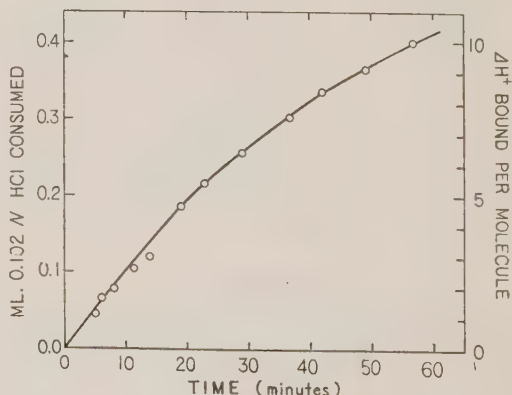


FIG. 3. Consumption of HCl for maintenance of a TAA solution (211 mg. TAA in 20.0 ml. of 0.1 *M* KCl) at constant pH (3.42) at 20° .

Consumption of Acid at Constant pH—Between pH 3 and 4.3, a pH shift was observed and continuous addition of hydrochloric acid was required to maintain the TAA solution at a constant pH. In Fig. 3 data from one experiment are shown. The result suggests a progressive increase in the amount of hydrogen ion bound by TAA. The ordinate on the right side of Fig. 3 shows the increase in the amount of hydrogen ion bound per TAA molecule assuming that all the acid consumption was attributable to an increase in the hydrogen ion bound.

Titration Curves—Titration curves between pH 2 and 6.5 are shown in Fig. 4. An increase with time in the amount of hydrogen ion bound is seen between pH 3 and 4.3. In addition, it was found that a back-

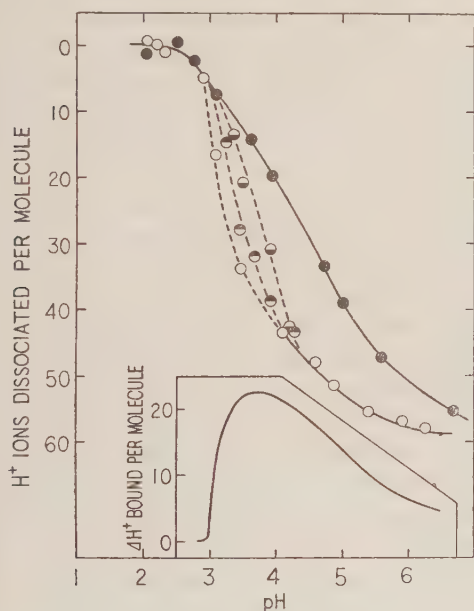


FIG. 4. Titration curves of TAA in acid pH region at 20°C in the presence of 0.1M KCl. Forward-titration (—○— after 4 minutes; —●— after 25 minutes; —●— after 100 minutes). Back-titration (—●— treated at pH 1.97 for 15 minutes at 20°C). The broken lines show the pH range where binding of H^+ ions proceeded at an observable rate. The difference in H^+ ions bound between 4 minutes and the back-titration data are shown as a function of pH in the insertion at the bottom of the figure.

titration from pH 1.97 (after maintenance at this pH for 15 minutes at 20°) deviates considerably from the forward-titration curve. Acid-treated TAA binds more hydrogen ions than non-treated TAA above pH 3. The difference between the forward-titration curve (4 minutes value) and the back-titration curve in the hydrogen ion bound is shown as a function of pH at the bottom of Fig. 4. No appreciable time-dependent change of binding was observed in the back-titration.

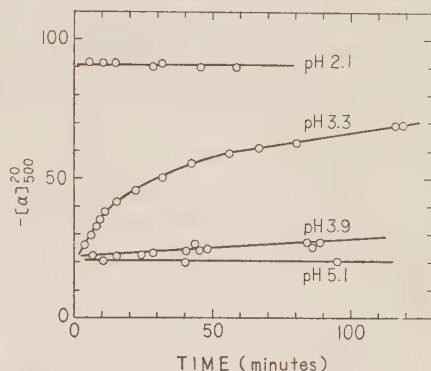


FIG. 5. Change in specific rotation of TAA with time.

Change of Optical Rotation with Time—The optical rotation measurement of TAA in acid pH region was designed to see the relation between the increase in hydrogen ion binding and the change in conformation of the TAA molecule. A progressive change in optical rotation was observed in the same pH region as the increase in hydrogen ion binding was observed. This suggests that the acid-denaturation and increase in hydrogen ion binding proceed simultaneously. It is also suggested that acid-denaturation occurs instantaneously below pH 3 and does not occur above pH 4.3, though in neither pH region was a change in hydrogen ion binding observed. Below pH 3 the denaturation and increase in binding might occur so rapidly that they could not be followed by the method used.

DISCUSSION

The data of Figs. 4 and 5 show that in the native state more than 20 ionizable groups

in TAA are apparently inaccessible to hydrogen ions and that they become accessible simultaneously with acid-denaturation. Judging from the pH region at which they are titrated, most of these groups seem to be carboxyl groups. These carboxyl groups must be present as carboxylate ions in a relatively stable form. The participation of the imidazole groups of histidine residues may be very slight, if any at all, since there are few such residues in TAA (7 residues per molecule (5, 6)) compared with the great number of acidic amino acid residues of a non-amide form (*c.a.* 40 residues per molecule (5, 6)) and since they have higher $pK_{\text{int.}}$ values (6.4–7.0) (7) than those of the carboxyl groups of the side chain (4.3–4.7) (7).

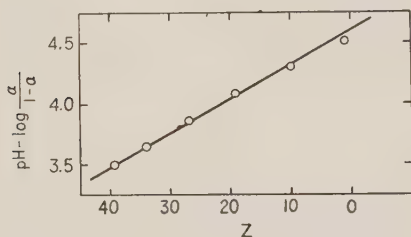


FIG. 6. Logarithmic plot of the back-titration data in Fig. 1 according to equation (2).

Regarding the hydrogen ion equilibria in acid-treated TAA as reversible, the titration data may be interpreted in terms of the following equation (7),

$$\text{pH} - \log \frac{\alpha}{1-\alpha} = pK_{\text{int.}} - 0.868wZ \quad \text{Eq. (2)}$$

where α is the degree of ionization of the groups concerned, Z represents the net charge of protein molecule, $pK_{\text{int.}}$ is the intrinsic dissociation constant of the groups and w is an empirical electrostatic interaction factor. Fig. 6 shows plots of $\text{pH} - \log \frac{\alpha}{1-\alpha}$ against Z , the value of Z being obtained from titration curves assuming that there is no binding of salt ions (the isoionic point of acid-denatured TAA was estimated as 5.1 from Fig. 4.). Fig. 6 gives a straight line and w and $pK_{\text{int.}}$ were determined from its slope and the intercept at $Z=0$. Its linearity indicates that no appre-

ciable change occurs in size, shape and permeability of the acid-treated TAA molecule over this pH range. It is also suggested that the ionization properties of the acid-treated TAA molecule are not influenced by the formation of a precipitate between pH 3 and 5.5. The values obtained are $w=0.032$ and $pK_{\text{int.}}=4.6$. The observed value of w is smaller than that (0.048) expected for the native TAA molecule (7). This indicates that the ionization behavior of the acid-treated TAA molecule approaches that of a typical flexible polyelectrolyte, but the relatively small decrease in w suggests that no drastic conformation change occurs in TAA on acid-treatment*. In β -lactoglobulin the value of w decreased to about one-tenth on alkali-denaturation (8, 9). The carboxyl groups of acid-treated TAA have a $pK_{\text{int.}}$ value which agrees well with the values (4.3–4.7) obtained for the carboxyl groups of other proteins in which the carboxyl groups ionize normally (7). This suggests that masked carboxylate groups in TAA begin to behave normally only after the TAA molecule is acid-denatured.

Recently Tanford (10) has pointed out that such an increase in hydrogen ion binding can be interpreted in terms of a change of the ionizing properties as a result of denaturation. The effect of denaturation on the ionizing properties is represented in theoretical treatment as a decrease in w . With TAA, however, no such interpretation is possible, because the increase in binding is remarkable around the isoionic point where the effect of the change in w is negligible (thus the significance of the second term in the right side of the equation (2) becomes negligible). In addition, the decrease in w on acid-denaturation of TAA is relatively small, as described above. So it is very probable that the increase in binding is the result of an unmasking of carboxylate groups which were inaccessible

* Although the results of optical rotation measurement suggest that considerable conformation change occurs on acid-denaturation, it is found in a preliminary experiment that the change is relatively limited when compared with that encountered on urea-denaturation.

to hydrogen ions.

As was reported previously that about 20 phenolic hydroxyl groups per TAA molecule are in a masked state and ionize irreversibly above pH 10.5 (1). So the formation of intramolecular hydrogen bonds between phenolic hydroxyl groups and side chain carboxylate groups may explain the anomalous dissociation of these two kinds of groups in TAA. Though the phenolic hydroxyl-carboxylate hydrogen bond is supposed to be weaker than the hydrogen bond between two peptide groups (11), it is expected that the former is a stable intramolecular bond, if such bonds exist abundantly in a protein molecule and support each other. It is also suggested that this sort of intramolecular bond may play a part in maintaining the stable structure of TAA, inferred from many experimental results. This stability is demonstrated by its resistance to denaturation by surface active agent (12) and urea (13), and its relatively high optical rotatory dispersion constant (1, 14-16)). Judging from the inaccessibility of hydrogen ions to the masked carboxylate groups down to the low pH value at which acid-denaturation occurs, these groups may be buried in the protein molecule and the binding of the hydrogen ions to the masked groups near the surface of the protein molecule might initiate the acid-denaturation of TAA. The assumption that the unmasking of the groups initiates the acid-denaturation is favorable for the interpretation of the mechanism of the acid-denaturation of TAA, because denaturation occurs in the pH region near the isoionic point of TAA where the electrostatic repulsion force is minimum and is not likely to cause denaturation.

Similar unmasking of ionizable side chain groups as observed with TAA has been observed with phenolic hydroxyl groups in ovalbumin (17) and in ribonuclease (18, 19), carboxyl groups in pepsin (20), carboxylate groups in lysozyme (21), and carboxylate and/or imidazole groups in hemoglobin (22). TAA seems to be the first example of a protein in which unmasking of two kinds of groups which can form intramolecular bonds among themselves was observed. Further investigation is neces-

sary, however, to clarify the significance of the presence of the masked groups to the tertiary structure of TAA.

SUMMARY

1. The ionization behavior of Taka-amylase A(TAA) in the acid pH region was studied by pH titration. It was found that more than 20 of the 40 carboxyl groups in a TAA molecule are in a masked state as carboxylate ions and combine with hydrogen ions irreversibly below pH 4.3.

2. From measurement of the optical rotation, it was found that the acid-denaturation of TAA occurs simultaneously with the unmasking of the carboxylate groups.

3. These results, together with the previous observations on the abnormal ionization of phenolic hydroxyl groups in TAA, suggest that carboxylate-phenolic hydroxyl hydrogen bonds play a part in maintaining the stable structure of TAA.

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Synthesis of Formyl Phosphate

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(Received for publication, August 12, 1960)

In a preliminary communication (1), Takeda and Nakagawa reported the isolation of a new formate activating enzyme from *E. coli* and proposed the name, formokinase, for this enzyme. Formokinase catalyzes the formation of formohydroxamate in the presence of formate, ATP and hydroxylamine. In subsequent investigations (2), it was shown that during the reaction ATP was split to ADP and inorganic orthophosphate and not to AMP and pyrophosphate. In the absence of hydroxylamine, an unknown compound accumulated. This compound was not adsorbed on charcoal and reacted with hydroxylamine, forming formohydroxamate. From these facts, it was concluded that the actual intermediate in the formokinase reaction was formyl phosphate rather than formyl adenylate.

Therefore attempts were made to synthesize formyl phosphate chemically and, using synthetic formyl phosphate, it was established that formyl phosphate is the actual intermediate in formate activation (2). The present communication is concerned with the synthesis of formyl phosphate.

EXPERIMENTAL AND RESULTS

Materials—Formic acid (98–100 per cent purity) was obtained from Merck Co. Dibenzyl phosphochloridate was synthesized by the method of Atherton *et al.* (3). Anhydrous formic acid was prepared by drying formic acid (98–100 per cent purity) over anhydrous copper sulfate for a week and then distilling it.

Palladium-charcoal was prepared as follows: A suspension of 3 g. of acid-activated charcoal and 150 mg. of palladium chloride in 15 ml. of 0.1 *N* HCl was placed in a 300

ml. Erlenmeyer flask. After the addition of 150 ml. of distilled water, the flask was flushed continuously with hydrogen for 1 hour with stirring at room temperature. The palladium-charcoal formed was collected by filtration and washed twice with 50 ml. portions of methanol and then twice with 50 ml. portions of distilled water, and finally dried in a vacuum desiccator over phosphorus pentoxide for 24 hours. The whole preparation was used for one run of catalytic hydrogenation with dibenzyl formyl phosphate.

Other chemical compounds were purchased from commercial sources in Japan.

Analytical Procedures—Formohydroxamate was measured spectrophotometrically by the method of Schweet (4). Labile phosphate was determined by the procedure of Fiske and Subbarow (5).

Synthesis of Formyl Phosphate

A. Preparation of dibenzyl formyl phosphate—Dibenzyl formyl phosphate was prepared by the condensation of dibenzyl phosphochloridate with formic acid.

To 40 ml. of dry chloroform* in a 100 ml. three-necked flask, equipped with a thermometer, a small dropping funnel, and a drying tube, were added 0.8 ml. of anhydrous formic acid and 1.7 g. of triethylamine. A solution of 5 g. of dibenzyl phosphochloridate in 10 ml. of dry chloroform was placed in the dropping funnel. The apparatus should be thoroughly dry.

The flask was immersed in an ice-salt bath and the dibenzyl phosphochloridate solution was carefully added dropwise from the dropping funnel with continuous stirring over

* Treated with concentrated sulfuric acid and distilled from anhydrous calcium chloride.

a 10 minute period so as to maintain the temperature of the mixture below 0°C. When the addition was complete, the mixture was stirred for a additional 30 minutes at 0°—5°C. After the condensation reaction had been completed, the chloroform and the residual formic acid were removed under reduced pressure and a syrupy material was obtained. On adding 50 ml. of dry ether to this syrupy material, triethylamine hydrochloride was precipitated and separated by vacuum filtration. The triethylamine hydrochloride was then washed twice more with the same volume of dry ether and was then discarded. The ethereal filtrate and the washings were combined. Then the ether was removed by distillation and the entire residual material was used for the next step.

B. Preparation of formyl phosphate—Formyl phosphate was prepared by catalytic hydrogenation of dibenzyl formyl phosphate.

A mixture of dibenzyl formyl phosphate, 50 ml. of anhydrous formic acid, and palladium-charcoal was placed in a hydrogenation of dibenzyl formyl phosphate was continued at 0°C until no more hydrogen was absorbed. Usually this reaction took about 5–6 hours. At the conclusion of the hydrogenation, insoluble materials were vacuum-filtered and discarded. The filtrate was transferred to a 200 ml. round-bottomed flask and subjected to vacuum distillation to remove completely the formic acid and the toluene formed. The resulting syrupy material was extracted with ether to remove residual dibenzyl formyl phosphate and dried in a vacuum desiccator over phosphorus pentoxide.

Assay of the preparation for hydroxamate formation indicated a yield of 56 per cent of the theoretical value, based on the amount of formic acid used. In the present method, it should be noted that water is excluded throughout the preparation procedure.

Identification of Formyl Phosphate—A portion of the product was treated with an excess of hydroxylamine and applied to an ascending paper chromatograms on Toyo-Roshi No. 51 paper, using several solvent systems. Subsequent color development of the hydroxamate

with FeCl_3 in 0.1 *N* HCl revealed a single spot having an R_f of 0.60 in water-saturated *tert*-amyl alcohol, 0.43 in water-saturated butanol, 0.11 in butanol:methyl ethyl ketone: ammonia: water (5:3:1:1), and 0.05 in butanol: ammonia: water (5:1:1). These R_f values were consistent with those of authentic formohydroxamic acid.

The purity of synthetic formyl phosphate cannot be easily determined because this compound is hygroscopic and unstable. However, since a major contaminant of the synthetic compound seems to be inorganic orthophosphate, it seems reasonable to consider that the ratio of labile phosphate to total phosphate reflects its purity. Therefore a portion of the formyl phosphate was diluted with an adequate volume of distilled water at 0°C and labile phosphate, total phosphate and formohydroxamate measured in aliquots of the solution. Table I gives typical data of these analyses and shows that, according to phosphate analysis, the purity is 75 per cent. However the actual purity of the product was probably higher than this because some formyl phosphate was destroyed during the assay procedure. Table I also shows that the ratio of labile phosphate to active formyl groups in the preparations is 1:1. Thus synthetic formyl phosphate contains one mole of phosphoric acid and one mole of formic acid.

TABLE I
Analytical Data of Formyl Phosphate

	Micromoles in 0.5 ml. aliquot	Purity ¹⁾ (per cent)
Labile phosphate ²⁾	15.1	75
Total phosphate ²⁾	20.0	
Formohydroxamate formed ³⁾	14.8	

1) $\frac{\text{Labile phosphate}}{\text{Total phosphate}} \times 100$

2) Estimated by the method of Fiske and Subbarow (5).

3) Estimated by the method of Schweet (4).

Stability of Formyl Phosphate—Table II summarizes the hydrolysis characteristics of formyl phosphate over a wide range of pH values and at different temperatures.

TABLE II

Hydrolysis Characteristics of Formyl Phosphate

Temperature	Incubation time (hours)	pH	Per cent hydrolysis ¹⁾
0°	24	1	61
		7	85
		9	100
50°	1	1	71
		7	100
		9	100
		anhydrous	5

- 1) FHX formed after incubation
 FHX formed from anhydrous formyl phosphate $\times 100$
 FHX: Formohydroxamate

Formyl phosphate is stable under anhydrous conditions. However, in the presence of water it decomposes rapidly, especially at an alkaline pH. The characteristic that it is more stable at an acidic pH than at an alkaline pH, resembles that of other carboxyl phosphates and of amino acyl adenylates.

DISCUSSION

A general method for the preparation of the carboxyl phosphate of fatty acid is based on the fact that the anhydride of fatty acids readily acylate orthophosphate in aqueous pyridine solution. Thus, acetyl phosphate can easily be prepared by the reaction of acetic anhydride with orthophosphate. However, this method cannot be applied to the synthesis of formyl phosphate because there is no anhydride form of formic acid.

The following methods for the preparation of carboxyl phosphate also failed for the present purposes.

1. Condensation of formic acid with orthophosphate in the presence of dicyclohexyl carbodiimide.

2. Reaction of amidophosphate with formic acid.
3. Application of the Gattermann-Koch reaction.

Greenberg *et al.* (6) reported that a compound having the properties of formyl phosphate can be prepared by phosphorolysis of the mixed anhydride of acetic and formic acids. However, in our experiences, this method is not satisfactory because the major product of the reaction is acetyl phosphate rather than formyl phosphate and it is very difficult to separate these two acyl phosphates.

Preparation of the dilithium- or sodium-salt of formyl phosphate was unsuccessful because of the instability of this compound.

SUMMARY

A method for the synthesis of formyl phosphate is described. The method consists of two steps: the first is the condensation of dibenzyl phosphochloridate with formic acid and the second, the catalytic hydrogenation of dibenzyl formyl phosphate.

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Sulfur Metabolism in Higher Plants

III. Further Studies on Sulfate Reduction in Excised Leaves

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The biosynthetic pathway of sulfur-containing amino acids from sulfate remains yet unknown. Particularly, only a few limited information is available in the literature concerning biochemical studies on this pathway in higher plants. In the previous papers we have reported a reduction of sulfate to sulfite and a formation of sulfide from sulfite in excised leaves of mung beans, and have suggested that cysteine may be synthesized from sulfate by passing through either sulfite and sulfide or sulfite and cysteinesulfinic acid (1, 2, 3). Fromageot and Perez-Milan demonstrated the reduction of sulfate to sulfite in intact tobacco leaves and the stimulative effect of light on the reduction was also revealed (4, 5).

Recently, both Bandurski's group (6, 7, 8) and Hilz and his coworkers (9, 10) demonstrated the reduction of sulfate to sulfite or sulfide in cell free extract of yeast and showed that 3'-phospho-5'-adenosinephosphosulfate was an intermediate in the process of the reduction, although 5'-adenosinephosphosulfate was found to be an intermediate in the case of *Desulfovibrio* (11, 12, 13, 14). All of these finding imply that the first step in the biosynthesis of cysteine from sulfate is the reduction of sulfate to sulfite. Squires and Bandurski found the presence of an enzyme system activating sulfate in an acetone powder extract of spinach leaves*, and strengthened the view that an intermediate in the reduction of sulfate to sulfite in plant tissues may be 5'-adenosinephospho-

sulfate.

The present paper deals with some further observations on sulfate reduction system in intact plant leaves.

Materials—Mung beans were grown on soil supplemented with $(\text{NH}_4)_2\text{SO}_4$, Na_2HPO_4 , and KCl. Throughout the entire period of plant growth, temperature was maintained at 20° to 30°C and 3,000 to 4,000 Lux day-light lamp was used as a light source. Secondary and tertiary leaves were picked up after 2 to 4 weeks of seeding and were used for experiments. Sometimes spinach and parsley leaves purchased from market were also used.

Infiltration of S^{35} -labeled Sulfate and Other Anions into Excised Leaves— S^{35} -Labeled sulfate and other anions such as molybdate and sulfite ions were infiltrated into excised leaves as described in the previous paper (1). The leaves were then incubated for 2 hours at 30°C under artificial 3,000 Lux light. In the experiments under the dark condition, an incubating vessel was sheltered with tinned paper.

Fractionation of S^{35} -labeled Compounds—The incubated leaves were washed five times with water and then were homogenized with 2 ml. of 10% KOH. Thirty mg. of Na_2SO_3 was added to the media as carrier. The homogenate was mixed with 10 ml. of 4N HCl in the presence of N_2 gas, and acid-volatile compounds in the homogenate were separated from nonvolatile compounds by bubbling with N_2 gas and trapped into 10 ml. of 1% KOH.

Seventy mg. of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ were added to the acid-volatile fraction, and a half portion of the mixture was assayed for radioactivity of total acid-volatile compounds. To the other half of it was added 5 ml. of 5% $\text{Sr}(\text{NO}_3)_2$ to precipitate sulfite. The insoluble material was separated by centrifugation, and was finally dissolved in dilute HCl. The above procedures were repeated. This fraction may contain only sulfite and was subjected to the radioactivity measurement after oxidation to sulfate by iodine.

* Squires, R., and Bandurski, R. S., private communication.

Silver nitrate or zinc acetate was added to the residual fraction of strontium treatment to precipitate sulfide, and the radioactivity of the precipitate was measured.

RESULTS AND DISCUSSION

Our previous paper has demonstrated the formation of S^{35} -labeled sulfite from S^{35} -labeled sulfate in excised leaves of mung beans infiltrated with both S^{35} -labeled sulfate and non-labeled sulfite (2). However, it was observed in this experiment that some radioactive acid-volatile compounds were formed from S^{35} -labeled sulfate even in the absence of non-labeled sulfite (Table I). The results indicate also that sulfite affects the reaction as a function of its concentration. Whereas sulfite at low concentrations ($3\text{--}6 \times 10^{-3}M$) exhibited the marked stimulating effect on the formation of acid-volatile compounds from sulfate, the effect at the higher concentration ($3 \times 10^{-2}M$) was less pronounced and more or less the same level as that of control was seen. Stimulating effect of sulfite on the formation of acid-volatile compounds from sulfate at the moderate concentration may be explained by the trapping of S^{35} -labeled sulfite formed from S^{35} -labeled sulfate, however, at the high concentration sulfite may cause the inhibition of sulfate reduction itself.

As shown in Table II, the formation of acid-volatile compounds from sulfate was also inhibited by the simultaneous infiltration of molybdate with S^{35} -labeled sulfate. Since it was found by Wilson and Bandurski (15) that molybdate inhibited the sulfate-activating enzyme in the cell free extracts of microorganisms, the present observation may indicate the operation of this enzyme in the mung bean tissues, and the intermediary formation of 5'-adenosinephosphosulfate during the conversion of sulfate to acid-volatile compounds might be implied.

Experiments on the fractionation of acid-volatile compounds have led to the conclusion that they are sulfite and sulfide. Table III shows the radioactivity measurement of both compounds after infiltrating S^{35} -labeled sulfate

TABLE I

The Effect of Sulfite on the Formation of Acid-Volatile Compounds

Exp. I	sulfate absorbed $\times 10^4$ c.p.m.	acid-volatile compounds c.p.m.	incorporation ratio per cent
none	152	8840	0.56
$3 \times 10^{-3}MSO_3^-$	44	14940	3.42
$6 \times 10^{-3}MSO_3^-$	32	11490	3.62
$3 \times 10^{-2}MSO_3^-$	18	1783	0.98
Exp. II			
none	102	3250	0.32
$3 \times 10^{-3}MSO_3^-$	34	5130	1.50
$6 \times 10^{-3}MSO_3^-$	27	2900	1.08
$3 \times 10^{-2}MSO_3^-$	24	1295	0.55

The secondary leaves of mung bean were used for Exp. I. and the tertiary leaves for Exp. II.

TABLE II

The Effect of Molybdate on the Formation of Acid-Volatile Compounds

	sulfate absorbed $\times 10^4$ c.p.m.	acid-volatile compounds c.p.m.	incorporation ratio per cent
none	64	4640	0.73
$3 \times 10^{-5}MMoO_4^-$	53	2952	0.55
$3 \times 10^{-4}MMoO_4^-$	82	1658	0.20
$3 \times 10^{-3}MMoO_4^-$	53	882	0.17

The tertiary leaves of mung bean were used for this experiment.

into the excised leaves. It would be noteworthy that two compounds were labeled when only S^{35} -labeled sulfate was infiltrated, however, the simultaneous infiltration of non-labeled sulfite with S^{35} -labeled sulfate resulted in the formation of S^{35} -labeled sulfite alone. This result may indicate that sulfide is formed from sulfate through sulfite. Thus the results in Table III may indicate the operation of sulfite reductase system in the leaves in conformity with our previous paper (3).

Light stimulation of sulfite formation from sulfate in tobacco leaves revealed by Fromageot's group was also observed in the formation of acid-volatile compounds in

mung bean, spinach and parsley leaves (Table IV). This observation strongly indicates that there exists a close relationship between the sulfate reduction system and the function of chloroplast. From the over-all

catalysed by chloroplast.

TABLE III

Analysis of Acid-Volatile Sulfur Compounds

Exp. No.	additions	total acid-volatile compounds c.p.m.	sulfite ⁽¹⁾ fraction c.p.m.	sulfide ⁽²⁾ fraction c.p.m.
I	none	1293	757	197
	$3 \times 10^{-3} \text{MSO}_3^-$	597	573	20
II	none	1542	821	326
	$3 \times 10^{-3} \text{MSO}_3^-$	622	560	28

Spinach leaves were used for this experiment.

1) Sulfite fraction shows the radioactivity of strontium precipitated compound.

2) Sulfide fraction shows the radioactivity of the compounds which is not precipitated by strontium but precipitated by silver or zinc ions. Silver nitrate was used for Exp. I., and zinc acetate for Exp. II.

TABLE IV

The Effect of Light on the Reduction of Sulfate to Sulfite

Exp. No.	sulfate absorbed $\times 10^4$ c.p.m.		acid-volatile compound c.p.m.		incorporation ratio per cent	
	light	dark	light	dark	light	dark
I.	39	31	6380	2440	1.65	0.78
II.	34	30	6425	1990	1.88	0.66
III.	27	25	9250	623	3.48	0.24
IV.	32	33	13650	2620	4.31	1.14
Spinach	63	68	1950	688	0.31	0.10
Parsley	37	31	1230	307	0.34	0.10

The incubation mixture contained $17\mu\text{C}$ of carrier free $\text{S}^{35}\text{O}_4^{2-}$ and $3 \times 10^{-3} \text{MNa}_2\text{SO}_3$. The secondary and tertiary leaves of mung bean were used for Exp. I-IV.

experimental results presented in this paper we can conclude that (1) an enzymatic sequence of sulfate reduction is operating in the plant leaves (sulfate \rightarrow active sulfate \rightarrow sulfite \rightarrow sulfide), and (2) all or part of the system is connected with the photochemical reaction

SUMMARY

Some properties of sulfate reduction system in higher plant tissues were investigated by the incorporation experiments using S^{35} -labeled sulfate.

Sulfate reduction in the leaves was inhibited by molybdate ion. Addition of non-labeled sulfite stimulated the reduction, but the stimulating effect was declined by increasing the concentration of sulfite.

S^{35} -Labeled product of sulfate reduction was shown to be sulfite in the presence of non-labeled sulfite, but both sulfite and sulfide were produced in the absence of sulfite.

Sulfite formation from sulfate in the leaves was activated with light.

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Studies on the Biosynthesis of Glycine in the Silkworm

I. Formation of Glycine from Serine*

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Glycine is known to be the major constituent amino acid of the silk fibroin of *Bombyx mori* and is actively synthesized in the silkworm at the fifth instar stage (1). The biosynthetic pathway of this amino acid in the silkworm, however, has hitherto not been clarified with certainty. Glyoxylic acid, which is contained on a relatively high level in the body fluid of the silkworm (2), was shown to be one of precursors of glycine (1, 3), and aminomalonic acid was also suggested to be the one (4, 5), but it is not yet certain whether these substances actually play a role as precursors in the biosynthesis of glycine in the silkworm.

It has generally been established that serine is the most important source of glycine in the mammalian body (6, 7). Although serine-glycine interconversion reaction was suggested to occur in the silkworm (8), direct evidence of the conversion of serine to glycine has so far been insufficient. Recently, however, Fukuda showed the incorporation of the radioactivity of C^{14} -labeled serine into glycine of cocoon fibres (9).

The present paper deals with an experiment in which serine-1- C^{14} was injected into silkworms and amino acids in the body fluid and the posterior silk-gland were isolated to measure their radioactivities. Each sample was taken at 20, 50, and 90 minutes after injection of labeled serine. It was observed that serine was rapidly utilized to a considerable extent

for the glycine biosynthesis in the silkworm.

EXPERIMENTALS

Silkworms.—Silkworms (*Bombyx mori* L., a hybrid, "Nichi 122×Si 122") were kindly supplied from the Experimental Sericultural Station of Miyagi Prefecture at the beginning of the fifth instar stage.

Preparation of Serine-1- C^{14} .—Serine-1- C^{14} was synthesized from glycine-1- C^{14} (purchased from the Radiochemical Center, Amersham, England) and formaldehyde with partially purified rat liver serine aldolase according to Alexander *et al.* (10). The labeled serine was purified by Dowex 50 column chromatography and had a specific activity of 1.56×10^5 c. p. m. per μ mole.

Injection of Serine-1- C^{14} .—Five hundredth ml. of 0.154 M KCl solution containing 4.0×10^4 c.p.m. of serine-1- C^{14} was injected into each silkworm at the sixth day of the fifth instar stage. Each experimental group consisted of ten silkworms.

Isolation of Amino Acids.—The body fluid of the silkworm was collected by puncturing one of their legs with a needle at definite intervals of time after injection. Then the silk-glands were immediately removed from the worms in the cold 0.154 M KCl solution. The body fluid was deproteinized by the addition of one third volume of 30 per cent perchloric acid. After removing excess perchloric acid as potassium perchlorate, the supernatant was subjected to a column chromatography of Dowex 50(H). The amino acids adsorbed on the resin were eluted from it with 2 N ammonium hydroxide. After removal of ammonium hydroxide, acidic amino acids were separated from neutral and basic amino acid fraction by adsorption on Amberlite IR-4B(Cl). Fractionation of the neutral and basic amino acids was carried out by the method of Hirs *et al.* (11).

The protein of the posterior silk-gland including silk fibroin was hydrolyzed with 6 N hydrochloric acid for 20 hours at 105°C in a sealed tube, and the amino

* A part of this study was presented at the Meeting of the Agricultural Chemical Society of Japan held in Tokyo on April 8, 1959.

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acids were analyzed by the same procedure as described above.

Measurement of Radioactivity—Radioactivities of amino acids and proteins were measured with a end-window type Geiger-Müller counter. Suitable corrections were made for background and self-absorption.

Determination of Serine and Glycine—The method of Frisell *et al.* (12) and Krueger (13) were employed respectively.

RESULTS

Radioactivities of Body Fluid and Silk-glands—Time course of the change in radioactivities of the body fluid and silk-glands is given in Fig. 1. A rapid decrease in the activity of the soluble fraction of the body fluid and an increase in the activity of the posterior silk-gland were observed, while only a little radioactivity was incorporated into the proteins of the middle silk-gland and the body fluid. A similar result on the distribution of C^{14} in various tissues of silkworm was previously reported from this laboratory in an experiment with glycine-1- C^{14} (8).

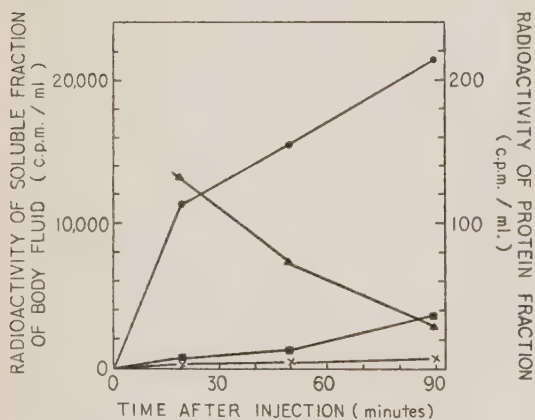


FIG. 1. Incorporation of serine-1- C^{14} into body fluid and silk-glands.

—▲— Soluble fraction of body fluid, —●— Protein fraction of posterior silk-gland, —■— Protein fraction of middle silk-gland, —×— Body fluid protein.

Distribution of Radioactivity of Amino Acids in Body Fluid—Most of the radioactivity of the amino acid fraction of the body fluid was found in a neutral and basic amino acid frac-

tions, of which only three amino acids, *i. e.*, serine, glycine and alanine were labeled with C^{14} (Table I). The specific activities (c.p.m.

TABLE I

Distribution of Radioactivity among the Amino Acids in the Body Fluid of the Silkworm Injected with Serine-1- C^{14}

Amino acid	Radioactivity (c.p.m./ml.)		
	Time after injection (minutes)		
	20	50	90
Neutral amino acid fraction	16,100	7,700	2,100
Serine	9,600	3,850	1,200
Glycine	4,900	2,240	500
Alanine	200	128	100
Threonine	0	0	0
Valine	0	0	0
Leucine	0	0	0
Isoleucine	0	0	0
Tyrosine	0	0	0
Phenylalanine	0	0	0
Basic amino acid fraction	0	0	0
Acidic amino acid fraction	0	0	0

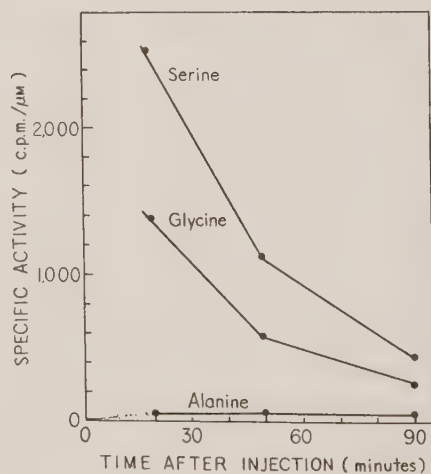


FIG. 2. Changes in the specific activities of free serine, glycine and alanine in the body fluid.

per mole) of these amino acids are shown in Fig. 2. A parallel decrease in the specific activities of serine and glycine was observed,

the specific activity of glycine being about 60 per cent of that of serine throughout this period. This fact suggests that a considerable part of free glycine in the body fluid may be derived from serine.

It is noted that a little but appreciable radioactivity was found in alanine.

Radioactivities of Amino Acids Isolated from Posterior Silk-gland—Most of the radioactivity of the amino acids of the posterior silk-gland hydrolyzate was found in serine and glycine, but little in alanine, as given in Fig. 3. Similar to the result of the amino acids from the body fluid, no activity was detected in the acidic amino acid fraction. While the specific activity of serine showed only a slight change in this period, that of glycine increased gradually and approached to the value of

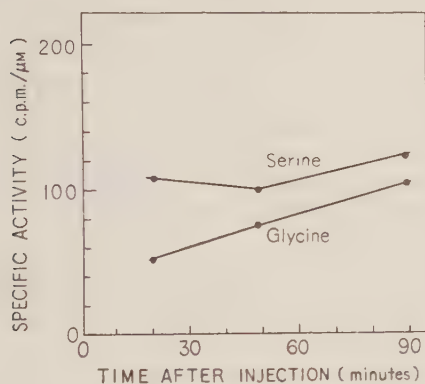


FIG. 3. Changes in the specific activities of serine and glycine in the posterior silk-gland protein.

TABLE II

Distribution of C¹⁴ in Glycine Isolated from Posterior Silk-gland

	Radioactivity (c.p.m./μmole)		
	Time after injection (minutes)		
	20	50	90
Glycine	56.9	75.7	100.8
COOH	57.5	77.0	103.0
CH ₂ NH ₂	0.0	0.0	0.0

serine. This fact indicates that free serine in the body fluid is effectively and rapidly utilized for the biosynthesis of silk fibroin.

Distribution of C¹⁴ in Glycine Molecule—The glycine isolated from the posterior silk-gland was degraded by the method of Vernon *et al.* (14). As shown in Table II, C¹⁴ was contained only in the carboxyl-carbon atom. This result indicates that the direct conversion of serine to glycine occurred in this period.

DISCUSSION

In the present work it was demonstrated that glycine was synthesized from serine in the silkworm, in good agreement with the results of Fukuda (9) obtained under different conditions.

The facts that the specific activity of glycine rose to nearly 60 per cent of that of serine by 20 minutes after injection (Figs. 2, 3) and the ratio of the specific activity of glycine to that of serine was approximately constant throughout this experiment (Fig. 2), indicate the importance of serine as a precursor of glycine in the silkworm as in the mammals (6, 7).

Recently it was shown in our laboratory that serine was the most radioactive among the amino acids isolated from the body fluid and the soluble fraction of the posterior silk-gland when uniformly labeled C¹⁴-glucose was injected into the silkworm (15). In addition, the formation of serine from 3-phosphoglyceric acid (16) and the interconversion of serine to glycine* were demonstrated enzymatically in the silkworm. These facts appear to be additional evidences supporting the assumption that serine is one of precursors of glycine in the silkworm.

Previously, it was suggested that glyoxylic acid may be an important precursor of glycine in the silkworm (1, 3). From the data presented here and our recent results from an experiment using glyoxylic acid-1, 2-C¹⁴ (17), serine seems to be more important precursor of glycine than glyoxylic acid. Details on the quantitative contribution of these two path-

* unpublished result

ways to the biosynthesis of glycine in the silkworm will be published later.

Alanine was observed to be labeled to a few but appreciable extent in the body fluid, but the mechanism by which serine is utilized for the synthesis of alanine is uncertain.

SUMMARY

1. The biosynthesis of glycine from serine was investigated in the silkworm injected with serine-1-C¹⁴. Glycine and serine were isolated from the body fluid and the posterior silk-gland at definite intervals of time after injection and the radioactivities of these amino acids were measured.

2. A considerable amount of the radioactivity of serine was observed to be incorporated into glycine both in the body fluid and in the posterior silk-gland.

3. The carboxyl carbon of the glycine isolated from the posterior silk-gland was exclusively labeled, showing the direct conversion of serine to glycine.

4. The significance of serine in the biosynthesis of glycine in the silkworm was discussed.

We are indebted to Mr. K. Noguchi, the chief of the Experimental Sericultural Station of Miyagi Prefecture, for his kind supply of the silkworm and to Mr. K. Otomo of our laboratory for his valuable assistance.

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Photochemical Phosphate Transfer in Green Leaves

II. Reconstruction of Reaction System

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It has been reported that on illuminating the homogenate of spinach leaves in the presence of an appropriate phosphate donor, such as glycerophosphate group of the substance was transferred to some endogenous acceptor (A) in the leaf to form a labile phosphoric compound (A~Ph).



The stoichiometry of the reaction and the nature of the endogenous acceptor in question were investigated in previous studies (1, 2). The present study deals with an attempt of separating the cellular components which participate in the process of the photochemical transphosphorylation.

MATERIALS AND METHODS

Preparation of Component Reactants: 1. Separation of grana—Sixty grams of washed spinach leaves were ground in a glass mortar with 60 ml. of *M*/50 acetate buffer, pH 6.0. The homogenate thus obtained was strained through cheese cloth and centrifuged first at a low speed ($5,000 \times g$, 5 minutes) to sediment cell debris and whole chloroplasts. The supernatant was then centrifuged at a higher speed ($20,000 \times g$, 15 minutes) and the precipitated *grana* were washed several times with an acetate buffer (*M*/50, pH 6.0), and finally suspended in 30 ml. of the same buffer. All preparatory procedures were performed at temperatures below 5°C to avoid loss of activity of the *grana*. The activity of photochemical transphosphorylation of the *grana* suspension was almost abolished within 24 hours, even when it was kept in a refrigerator at 0°C. The experiments were, therefore, performed immediately after the *grana* preparations were made.

2. Separation of cytoplasmic enzyme—Separation of the cytoplasmic enzyme required for the reaction under investigation was also carried out at temperatures below 5°C. Three hundred grams of spinach leaves were ground in a Waring blender with 300 ml. of *M*/50 acetate buffer, pH 6.0, and 100 g. of crushed ice. The homogenate obtained was strained through cloth, and pH adjusted to 4.5~4.6 by the addition of *N*/10 acetic acid. The precipitate formed, which included the greater part of the *grana*, was centrifuged off, and the supernatant was gradually adjusted to pH 6.0 with *N*/10 KOH. Cytoplasmic enzyme was prepared from this supernatant by ammonium sulfate fractionation (between 0.35~0.50 saturation). This fractionation procedure was repeated twice. Finally, the precipitate was dissolved in 30 ml. of acetate buffer (pH 6.0, *M*/50) and dialyzed against the same buffer in a refrigerator for 20 hours. The enzyme preparation thus obtained maintained its activity for about a week in a refrigerator at 0°C. Before use, the enzyme preparation was diluted 10~20 times.

3. Separation of endogenous phosphate acceptor—Four kilograms of spinach leaves were ground in a Waring blender with 3 liters of deionized water. The homogenate obtained was heated at 60°C for 10 minutes. The green sediment formed was filtered through cheese cloth and filter paper. Saturated solution of basic lead acetate was added to the filtrate until no more precipitate was formed from the supernatant. The yellowish precipitate was then separated by centrifugation, washed once with water, suspended in 500 ml. of water, and decomposed with hydrogen sulfide. Before decomposition, a very small amount of octyl alcohol was added to the reaction mixture to prevent vigorous foaming. The supernatant solution was gradually neutralized with *M*/10 NaOH to pH 6.0. To the dark brown solution thus obtained was added *M*/5 AgNO₃ solution until no greyish-white precipitate was formed from the supernatant. The precipitate containing the active component was separated by centri-

fugation, washed once, suspended in 30 ml. of water, then a saturated NaCl solution was added to detach the silver combined with the phosphate acceptor and AgCl formed was centrifuged off. Since the supernatant also contained a significant amount of inorganic phosphate, a part of this inorganic phosphate was removed by precipitation with 10 ml. of $M/10$ CaCl_2 (Addition of excess amounts of CaCl_2 resulted in a loss of the phosphate acceptor). The phosphate acceptor contained in the supernatant solution was precipitated by the addition of an equal volume of acetone. The precipitate was dried *in vacuo*, dissolved in 50 ml. of water and the solution was pass through a cation exchange resin column (Amberlite IRC 50). The uncombined effluent was then run through an anion exchange column (Amberlite IR 4B), and the anion absorbed was eluted from the resin with $N/10$ NaOH or NaCl and the solution was finally adjusted to pH 6.0 with $N/10$ acetic acid.

Measurement of photochemical transphosphorylation—The reaction could be followed in two ways; either (i) by using a phosphate donor labeled with P^{32} and measuring the radioactivity of the reaction product after separating it by paperchromatography, or (ii) by running light and dark experiments in parallel, and measuring the difference in quantity of inorganic phosphate liberated. The values obtained for phosphate liberated in the dark control represent the phosphatase activity of the enzyme preparation used. Since the former method is time-consuming and cumbersome, often giving erratic results owing to the lability of the reaction product, the latter method was exclusively used for the quantitative determination of the reaction.

The reaction was performed in Warburg type vessels (diameter, 6 cm.; volume, 50 ml.). Two milliliters of *grana* suspension, 1 ml. of cytoplasmic enzyme solution, 1 ml. of phosphate acceptor solution and 2 ml. of acetate buffer (pH 6.0; final concentration, $M/50$) were placed in the main compartment of the reaction vessel. The solution of phosphate donor was placed in the side-arm of the vessel to make a final concentration in the reaction mixture of $5.5 \times 10^{-3} M$.

After evacuation, the reaction was started by mixing the two component parts, and the reaction vessel was shaken under illumination. The light source was tungsten lamps giving an illumination of 6,000 lux as measured at the bottom of the vessels. A dark control was simultaneously run. At suitable intervals, 1 ml. aliquots of the reaction mixture were taken out into test-tubes containing 0.1 ml. of ice-cold 10% trichloroacetic acid. The acidified reaction mixture was suitably diluted with cold water, filtered through

an ash-free filter paper, and 1 ml. aliquot of the filtrate was used for the determination of inorganic phosphate which was performed according to Takahashi's modification (3) of the methods described by Martin and Doty (4), and Lowry and Lopez (5).

The determination of inorganic phosphate was carried out within 5 minutes after terminating the reaction with an acid solution at a temperature below 5°C , to minimize the decomposition of the photo-produced labile phosphate ester.

RESULTS AND DISCUSSION

Preliminary experiments were performed to test the pH- and temperature-dependence of the process of photochemical transphosphorylation.

Influence of pH—Figure 1 shows the effect of pH on the photochemical transphosphory-

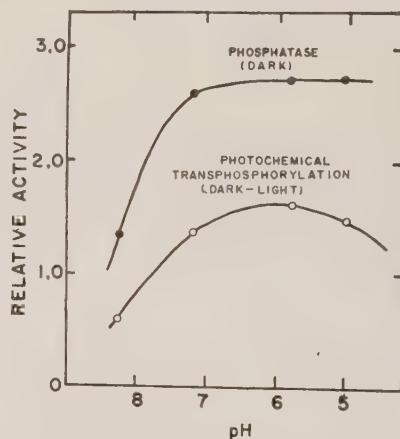


FIG. 1. Photochemical transphosphorylation and (acid) phosphatase reaction as influenced by pH.

Complete reaction system with *grana*, cytoplasmic enzyme, acceptor prepared from spinach and β -glycerophosphate (as phosphate donor and concentration: $5.5 \times 10^{-3} M$). Experimental conditions same as given in text, but with varied pH (veronal buffer).

lation. It may be seen that the optimum pH for this reaction lies at about 6.0. This is significantly different from that reported for the process of photosynthetic phosphorylation, indicating that the reaction observed here is different from that of photosynthetic phos-

phorylation. According to Arnon and his coworkers (6), the optimum pH for photosynthetic phosphorylation lies at about pH 8.0, and the reaction is undetectable at a pH as low as 6.0.

Influence of Temperature—Figure 2 shows the temperature-dependence of the photochemical transphosphorylation. The optimum

TABLE I

Photochemical Transphosphorylation in Presence of Various Substances as Phosphate Donors

Phosphate Donor		Ortho-Phosphate Liberated, after Incubation for 75 minutes ($\times 10^{-3}M$)	
		Light	Dark
Exp. 1	α -glycerophosphate	0.46	1.05
Exp. 2	β -glycerophosphate	0.83	3.38
Exp. 3	3-phosphoglyceric acid	0.92	4.24
Exp. 4	Glucose-1-phosphate	1.03	1.72
Exp. 5	Fructose-6-phosphate	2.01	5.03
Exp. 6	Fructose-1,6-diphosphate	2.52	6.74

Reaction mixture: 2 ml. *grana* suspension, 1 ml. acceptor solution, 1 ml. cytoplasmic enzyme, 2 ml. acetate buffer (final concentration, $M/50$; pH 6.0) and 1 ml. phosphate donor (final concentration, $5.5 \times 10^{-3} M$). Incubation temperature: $24^\circ C$; partial anaerobiosis (7 mm. Hg, air); illumination: 6,000 lux.

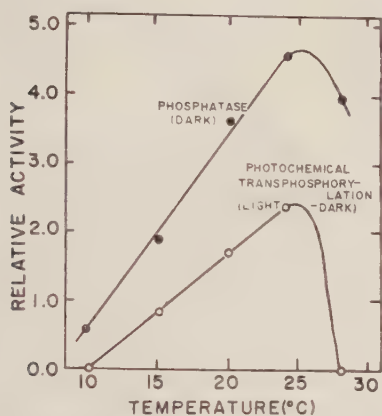


FIG. 2. Temperature dependence of photochemical transphosphorylation and (acid) phosphatase reaction.

Complete reaction system with β -glycerophosphate as phosphate donor (end concentration: $5.5 \times 10^{-3} M$). Experimental conditions same as given in text, but at varied temperatures.

TABLE II

Tests for Utilization of Inorganic Phosphate by the Reaction System of Photochemical Transphosphorylation
(control, with phosphoglyceric acid as source of phosphatase)

Incubation Time (min.)		Inorganic Phosphate in Reaction Mixture ($\times 10^{-3} M$)			
		0	30	60	90
Phosphate Sources added (Final Concentration)	Light				
	Dark				
None	Light	2.41	2.53	2.56	2.58
	Dark	2.41	2.54	2.58	2.57
Ortho-phosphate ($0.9 \times 10^{-3} M$)	Light	3.31	3.41	3.48	3.54
	Dark	3.31	3.39	3.50	3.51
Ortho-phosphate ($3.0 \times 10^{-3} M$)	Light	5.41	5.53	5.57	6.61
	Dark	5.41	5.54	5.57	6.65
Phosphoglyceric Acid ($5.5 \times 10^{-3} M$)	Light	2.41	2.72	3.12	3.34
	Dark	2.41	4.04	5.68	6.63

Experimental conditions same as in Table I.

lies at 24°C, and the reaction is not detectable below 10°C and above 28°C.

Based on these findings, all the following experiments of photochemical transphosphorylation were performed at pH 6.0 and at 24°C.

Phosphate Donor—The specificity of photochemical transphosphorylation was investigated with respect to the donor of the phosphate utilized in the reaction. The phosphate donors used in our original work (1) have been α - and β -glycerophosphates. Besides these a wide variety of phosphoric esters were found to be effectively utilized as phosphate donors. They include, as may be seen from the results shown in Table I and II, some intermediary low-energy phosphate compounds of photosynthesis (7), such as 3-phosphoglyceric acid, fructose-1,6-diphosphate, fructose-6-phosphate and glucose-1-phosphate. Noteworthy was the fact that, so far as the experimental conditions examined were concerned inorganic phosphate could not be directly utilized as phosphate donor (see Table II), a fact indicating that the process under investigation does not consist in an incorporation of inorganic phosphate liberated from the phosphate donor by the action of phosphatase, but that it represents a transfer of a phosphate group from the donor to the acceptor molecule. In the following experiments, the photochemical transphosphorylation was tested with 3-phosphoglyceric acid as donor.

Phosphate Acceptor—Figure 3 illustrates a typical experiment to show the indispensable role of the added endogenous phosphate acceptor. No reaction occurred when the acceptor was omitted from the complete reaction system. The specificity of the transphosphorylating system with regard to the acceptor was found to be extremely rigorous, all the substances thus far tested other than the endogenous substance in question, including AMP, UMP, ADP, RNA, DNA, riboflavin and various sugars, giving negative results in this respect. The chemical nature of the endogenous phosphate acceptor of spinach leaves will be described elsewhere.

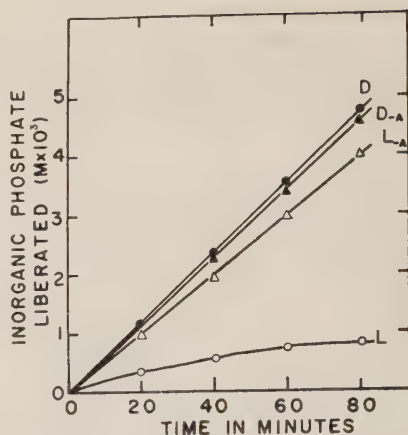


FIG. 3. Role of endogenous phosphate acceptor in photochemical transphosphorylation.

L, in light; D, in dark; subscript-A indicates the omission phosphate acceptor from reaction mixture. Reaction system and experimental conditions same as given in Table I. Phosphate donor: 3-phosphoglyceric acid (end concentration, $5.5 \times 10^{-3} M$).

Role of Grana—In view of the photochemical nature of the process, the indispensable role of the *grana* will readily be understood. In fact, if the *grana* were omitted from the complete reaction system (*grana*, cytoplasmic enzyme, donor and phosphate acceptor), no photochemical transfer of phosphate was obtained. The liberation of inorganic phosphate from the phosphate donor, effected by the cytoplasmic enzyme in the absence of added *grana*, was found to be unaffected by light (Fig. 4). The reaction rate of phosphate transfer in the complete system was found to be proportional to the amount of added *grana* (Fig. 5).

Role of Cytoplasmic Enzyme—If the cytoplasmic enzyme was omitted from the complete reaction system, there was no photochemical transfer of phosphate; both in the light and in the dark equally insignificant amounts of inorganic phosphate were liberated. Indispensability of the cytoplasmic enzyme was also shown by the paperchromatographic examination of the illuminated reaction mixture. No spot corresponding to the reaction product of photochemical transphosphorylation (see previous paper (2)) was discovered when

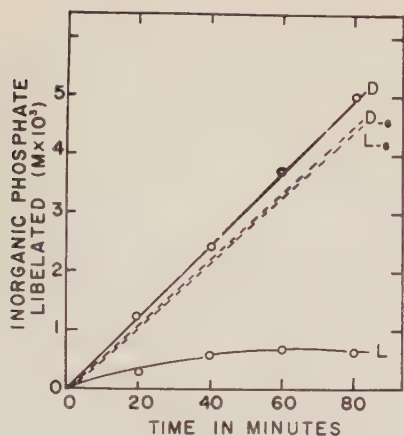


FIG. 4. Role of *grana* in photochemical transphosphorylation.

D, in dark; L, in light; subscript -G indicates omission of *grana* from the reaction mixture. Reaction system and experimental conditions same as given in Table I. Phosphate donor; 3-phosphoglyceric acid (end concentration, $5.5 \times 10^{-3} M$).

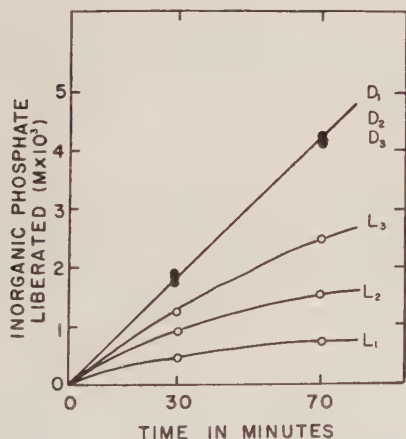


FIG. 5. Effect of concentration of *grana* on photochemical transphosphorylation.

L, in light; D, in dark; subscripts 1, 2 and 3 indicate the amounts of added *grana* in the reaction mixture (in terms of chlorophyll content: 0.24, 0.12 and 0.06 mg./ml., respectively.) Reaction system and experimental conditions same as given in Table I. Phosphate donor: 3-phosphoglyceric acid (end concentration, $5.5 \times 10^{-3} M$).

the enzymic component had not been added to the reaction mixture. It may also be worth noting here that the preparations of cytoplasmic enzyme prepared in the same way

from various sources, *i.e.*, green leaves of barley, *Brassica rapa* and spinach, were interchangeable with respect to their role in complementing the reaction system (Table III).

TABLE III
Photochemical Transphosphorylation with Cytoplasmic Enzyme from Various Sources (Green Leaves)

Sources of Enzyme (Green Leaves)	Inorganic Phosphate in Reaction Mixture ($\times 10^{-3} M$)			
	Initial	After Incubating for 60 Minutes		
		Light	Dark	Dark-Light
Spinach	2.40	3.14	6.36	3.22
<i>Brassica rapa</i>	2.40	5.61	6.65	1.04
Barley	2.40	6.13	7.29	1.16

Phosphate donor: 3-phosphoglyceric acid (final concentration: $5.5 \times 10^{-3} M$). Experimental conditions same as in Table I.

TABLE IV
Incapacity of Potato and Urine Enzymes in Replacing Cytoplasmic Enzyme of Green Leaves

Source of Enzyme	Inorganic Phosphate in Reaction Mixture ($\times 10^{-3} M$)			
	Initial	After Incubation for 40 Minutes		
		Light	Dark ¹⁾	Dark-Light
Spinach Leaves	0.69	2.13	3.87	1.74
Potato Tuber	0.69	3.66	3.66	0.00
Spinach Leaves	0.48	1.23	2.22	0.96
Human Urine	0.48	3.36	3.36	0.00

Phosphate donor: 3-phosphoglyceric acid (final concentration $5.5 \times 10^{-3} M$). Experimental conditions same as Table I.

1) Values for dark experiments represent (acid) phosphatase activities of the enzyme preparations used.

It should be remarked that all the preparation which displayed the activity of cytoplasmic enzyme also showed a high activity of acid phosphatase. The values for the optimum pH and optimum temperature for phosphatase activity coincided with those for photochemical transphosphorylation (Figs.

2 and 1). The two activities were found to be equally sensitive toward $M/1000$ 2, 4-dinitrophenol. These findings are suggestive of the possibility that the acidic phosphatase contained in the preparation of cytoplasmic enzyme is playing an essential role in the phosphate transfer under investigation. This, however, does not mean that any phosphatase can act as the catalyst in question. In fact, the phosphatase preparation from potato tubers and urine in the same way as above, could not replace the cytoplasmic enzyme of leaves, despite their high (acid) phosphatase activity (see Table IV). No conclusive evidence has yet been obtained to decide if this discordance was caused by unfitness of the tuber and urine enzyme proteins, or rather by the lack in these preparations of some co-factors necessary for the transfer reaction.

SUMMARY

1. Using homogenates of spinach leaves as material, investigations were made the process of photochemical transphosphorylation, in which phosphate from a low-energy phosphorous compound (donor, S-Ph) is photochemically transferred to an endogenous phosphate acceptor (A) to form an unidentified labile phosphorous compound (A~Ph)



2. The reaction system was found to consist of *grana*, cytoplasmic enzyme, phosphate donor and acceptor. These components, separately prepared, were united to reconstruct a complete reaction system. No reaction occurred when any one of these components was lacking in the reaction system.

3. The reconstructed reaction system of photochemical transphosphorylation utilizes as phosphate donor such substances as α - and

β -glycerophosphate, 3-phosphoglyceric acid, fructose-1, 6-diphosphate, fructose-6-phosphate, and glucose-1-phosphate. Inorganic phosphate is not utilized. The only substance thus far discovered to be utilized as phosphate acceptor is an endogenous substance which was extracted and partially purified from green leaves. Such substances as AMP, UMP, ADP, DPN, riboflavin and various sugars are ineffective in this respect.

4. The enzyme preparations obtained from green leaves of spinach, barley, and turnip (*Brassica rapa*), all highly active in (acid) phosphatase action, were found to be effective as cytoplasmic enzyme in the above reaction system. This could not be replaced by similar preparations of acid phosphatase prepared from potato tubers and urine. A possible role of acid phosphatase of green leaves in the process of photochemical transphosphorylation was suggested.

Authors express their gratitude to Prof. H. Tamiya for his guidance during this investigation. Authors also wish to thank Profs. A. Takamiya and Y. Ogura for their advices.

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Photochemical Phosphate Transfer in Green Leaves

III. Effect of Preillumination

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It has been previously shown (1, 2, 3) that the homogenate of spinach leaves contain a reaction system, by which phosphate from a low-energy phosphorous compound (phosphate donor) is photochemically transferred to an endogenous phosphate acceptor to form a certain labile phosphorous compound. The reaction system has been reconstructed, using *grana*, cytoplasmic enzyme and the endogenous acceptor separately prepared from green leaves, and an exogenous phosphate donors, such as 3-phosphoglyceric acid or glycerophosphate.

Recently it was discovered that preillumination of the reaction system caused the appearance of a capacity for transphosphorylation as detected by a subsequent test (in the dark) for the activity. The experimental results dealing with this phenomenon form the subject of this paper.

MATERIALS AND METHODS

Grana, cytoplasmic enzyme and the endogenous phosphate acceptor were prepared according to the procedures described in the foregoing paper (2).

Photochemical transphosphorylation was estimated as described previously, by measuring the differences in amounts of inorganic phosphate liberated from the reaction system in the light and in the dark. The reaction mixture containing 2 ml. *grana* suspension (chlorophyll content; 1.52 mg.), 1 ml. acceptor solution, 1 ml. cytoplasmic enzyme solution and 2 ml. acetate buffer (pH 6.0; final concentration, $M/50$) was placed in the main chamber of a Warburg type vessel. The phosphate donor, 3-phosphoglyceric acid (PGA; $5.5 \times 10^{-3} M$, final concentration), neutralized to pH 6.0 with NaOH, was placed in the side-arm of the vessel. The vessels (fitted with stopcocks) were connected to a manifold and evacuated simultaneously (7 mm Hg air).

The evacuated mixtures were incubated under continuous shaking in a water bath (24°C) for desired periods in the light (preincubation) or in the dark (control). The light source was a tungsten lamp giving an illumination of 6,000 lux as measured at the bottom of the reaction vessel. The phosphate donor solution in the side arm of the vessel was poured into the main chamber immediately after the light was turned off. At suitable intervals, 1 ml. aliquots of the reaction mixture were transferred into test tubes containing 0.1 ml. of cold 10% trichloroacetic acid.

The acidified reaction mixtures were then filtered through an ash-free filter paper, suitably diluted with cold water, and the amounts of inorganic phosphate in the aliquots were determined by the method described previously (2). Transphosphorylation was estimated by the difference in amounts of inorganic phosphate liberated in the light and in the dark (see below).

RESULTS

Effect of Preillumination—Figure 1 illustrates a typical result of experiment performed to estimate the effect of preillumination. Two parallel tests were run, the one preincubated for 15 minutes in the light (preillumination) and the other in the dark (control), and the liberation of inorganic phosphate was compared during and after this period of preincubation. A certain amount of inorganic phosphate was liberated during preincubation, but this endogenous reaction was found to be independent of illumination. At the end of the preillumination period, the phosphate donor (PGA) was added and both mixtures were incubated in the dark. In the non-preilluminated reaction mixture (control), there occurred an immediate and steady libe-

ration of inorganic phosphate, corresponding to the phosphatase activity of the reaction mixture in the dark (see previous paper). In the preilluminated reaction mixture, on the other hand, there was evidently an initial suppression of phosphate liberation, the same rate of endogenous phosphate liberation being maintained for the first 10 minutes. In view of the evidence obtained in the previous study of this series, (1, 3), this may be interpreted as a manifestation of the transphosphorylation induced by the preillumination of the reaction mixture.

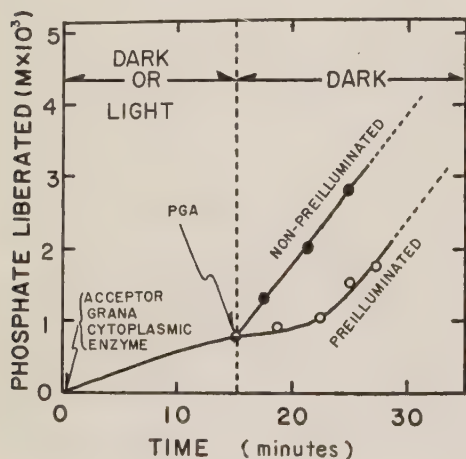


FIG. 1. Effect of preillumination on photochemical transphosphorylation.

Reaction mixtures in pretreatment contained: 2 ml. *grana*, 1 ml. acceptor, 1 ml. cytoplasmic enzyme and 2 ml. acetate buffer (pH 6.0; final concentration, $M/50$). After pretreatment in light or dark, 1 ml. 3-phosphoglyceric acid (neutralized to pH 6.0 with NaOH; final concentration, $5.5 \times 10^{-3} M$) were added in the dark. Reaction temperature: $24^\circ C$.

Accordingly, the preillumination effect was estimated by the difference in amounts of phosphate liberated in the preilluminated and non-preilluminated reaction mixtures on subsequent addition of PGA in the dark.

In the experiment reproduced in Fig. 2, the preincubation was made with mixtures of *grana* and the acceptor alone, and the cytoplasmic enzyme and PGA were added at the beginning of the subsequent dark period.

As may be seen, the preillumination effect was not affected by the absence of the enzyme during the preillumination period indicating the non-participation of cytoplasmic enzyme in this phase of the reaction. In the absence of cytoplasmic enzyme during the preillumination period the liberation of phosphate was negligibly small whether in the light or in the dark, a finding which was to be expected, since the phosphatase activity was contained only in this fraction of the reaction system (see previous report). No transfer of phosphate was obtained, if cytoplasmic enzyme was not added together with PGA in the subsequent dark period, indicating the essential role of the cytoplasmic enzyme in this step of the reaction. Omission of the acceptor from the reaction mixture during the preillumination period also resulted in an abolition of the preillumination effect.

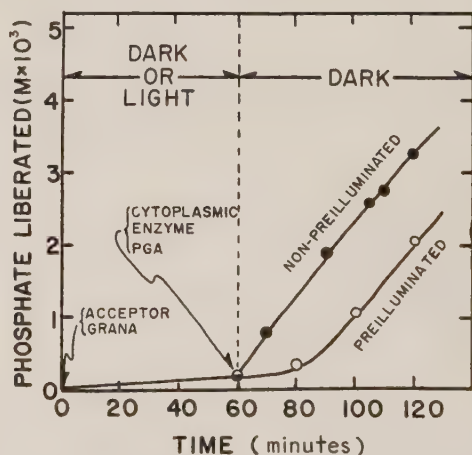


FIG. 2. Effect of preillumination on photochemical transphosphorylation (Preillumination in absence of cytoplasmic enzyme).

Reaction mixtures in pretreatment contained: 2 ml. *grana*, 1 ml. acceptor and 2 ml. acetate buffer (pH 6.0; final concentration, $M/50$). After pretreatment in light or dark, 1 ml. cytoplasmic enzyme and 1 ml. 3-phosphoglyceric acid (neutralized to pH 6.0 with NaOH; final concentration, $5.5 \times 10^{-3} M$) were added in the dark. Reaction temperature: $24^\circ C$.

Influence of Preillumination Time—Figure 3 illustrates the effect of preillumination as it increased with the duration of illumination.

In this experiment a mixture containing *grana*, cytoplasmic enzyme and acceptor was preilluminated varied period of 10 to 60 minutes and its effect was determined at the 30th minute of the subsequent dark incubation. As may be seen the capacity for transphosphorylation gradually increased with the time of preillumination to attain a maximum level after about 60 minutes of illumination.

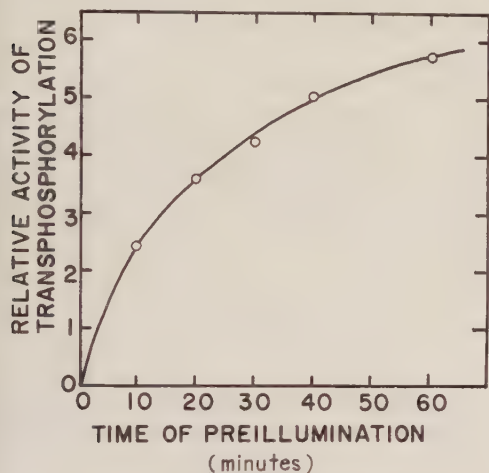


FIG. 3. Time-course of the development of preillumination effect.

Reaction mixture consisting of 2 ml. *grana*, 1 ml. phosphate acceptor, 1 ml. cytoplasmic enzyme and 2 ml. acetate buffer (pH 6.0; final concentration, $M/50$) was illuminated after various times indicated the light was turned off simultaneously with the addition of 1 ml. 3-phosphoglyceric acid (neutralized to pH 6.0; final concentration, $5.5 \times 10^{-3}M$). At the 30th minute of dark incubation the amounts of inorganic phosphate in the reaction mixture were determined, and they were compared with the blank values which were obtained with the samples not subjected to preillumination.

Duration of Preillumination Effect—The effect of preillumination was found to have a certain life-time, showing a decay in the dark when the photoactivated system was not in contact with its reactant. In the experiment reproduced in Fig. 4, a mixture containing *grana*, cytoplasmic enzyme and acceptor was first subjected to preillumination for 60 minutes, and after turning off the light (under anaerobic conditions), PGA was added at

different times to the mixture. Determination of transphosphorylation was performed 10 minutes after the addition of PGA. As may be seen from the figure the light-induced capacity of the illuminated system disappeared within 15 minutes when not in contact with PGA. This result suggests the unstableness of the substance (or state) produced by the preillumination.

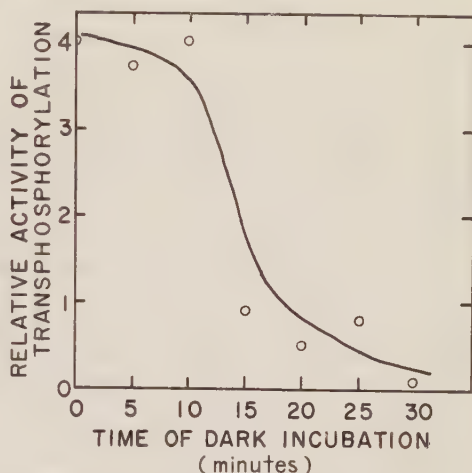


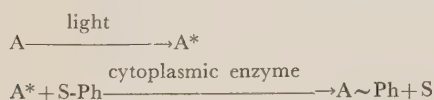
FIG. 4. Spontaneous decay of preillumination effect in subsequent dark-incubation.

Mixture containing *grana* and acceptor was preilluminated for 60 minutes; and after varied periods of subsequent dark-incubation, PGA and cytoplasmic enzyme were added. Preillumination effect (transphosphorylation induced) was measured by difference in amounts of inorganic phosphate liberated in preilluminated and non-preilluminated reaction mixtures during 10-minute reaction period (in dark) after addition of PGA.

DISCUSSION

The ensemble of experimental results described above definitely indicates that the process of photochemical transphosphorylation under investigation consists of two separate reaction-steps, namely, an initial light-requiring reaction and a subsequent dark-reaction, and that among the four essential components of the reaction system, *i. e.*, *grana*, endogenous phosphate acceptor, cytoplasmic enzyme and phosphate donor, (see previous paper (3)), the first two are involved in the light-process,

while the third and fourth participate in the dark-reaction. Conceivably the mechanism of the light-reaction may be a conversion of the endogenous principle into an active substance which will serve as the actual acceptor of phosphate in the subsequent transfer of phosphate in the dark. The sequence of events may thus be formulated as follows:



where A and A* represent, respectively, the endogenous principle and its photoactivated form functioning as the phosphate acceptor. S-Ph is the donor of phosphate, and A~Ph the reaction product.

Now, the most important points to be made clear are the chemical nature of A and A* and the process of the conversion of the former into the latter. The possibility of an oxido-reductive process taking part in this change have to be examined. There exists also a possibility that the acceptor designated as A above, actually consists of two parts, a true acceptor, and a cofactor, the light exerting its effect by converting one of them into its active form. Further investigations along these lines are in progress in parallel with the attempts to purify and identify the assumed phosphate acceptor.

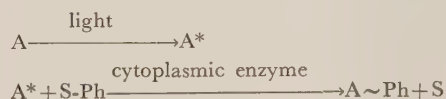
SUMMARY

1. Photochemical transphosphorylation in green plants was investigated, using preparations of each reaction components isolated from spinach leaves, *i. e.*, *grana*, cytoplasmic enzyme and endogenous acceptor, as well as phosphate donor such as 3-phosphoglyceric

acid.

2. Preillumination of endogenous acceptor in the presence of *grana* was found to induce a capacity for transferring phosphate (in the dark) on subsequent addition of phosphate donor and cytoplasmic enzyme.

The time course of development and decay of the light-induced capacity during preillumination and subsequent dark period was investigated. The light-induced capacity for transphosphorylation was found to increase with time to attain a maximum level after 60 minutes of preillumination, and to decrease rapidly on subsequent dark incubation, the activity being totally abolished in 15 minutes in the dark. The following reaction scheme is produced to account for the experimental results obtained:



where A and A* represent, respectively, the endogenous and active forms of phosphate acceptor. S-Ph is the donor of phosphate and A~Ph the reaction product.

Authors express their gratitude to Prof. H. Tamiya for his guidance during this investigation.

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Studies on the Biosynthesis of Silk Fibroin

III. In vivo Incorporation of Glycine- C^{14} into Proteins of Posterior Silkland Fractions*

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In studying protein biosynthesis, it is a fundamental problem to investigate the cellular structures performing the synthesis of the protein being studied. Silk fibroin is a protein secreted into the cavity of the posterior silkland (1). The gland is known to be strongly basophilic throughout the cytoplasm and to secrete fibroin as the only physiological function. When the posterior silklands were fractionated in 0.25 *M* sucrose according to Schneider (2), large granule fraction was most actively incorporated with glycine-1- C^{14} (3, 4) and had the highest succinoxidase activity (3). This was a peculiar point for silklands. As Simpson *et al.* (5) reported that labeled leucine incorporation into mitochondrial proteins was in nearly the same magnitude as into microsomal proteins in skeletal muscle cells, we have brought forward a problem whether the both activities are linked to the same cellular structure or not.

In this paper it is shown that glycine-incorporating activity could be separated from succinic dehydrogenase activity by differential centrifugation. And using an improved fractionation procedure, glycine-1- C^{14} incorporation *in vivo* into the silkland fractions was investigated.

MATERIALS AND METHODS

Silkworms—Domestic silkworms, *Bombyx mori* L., at the third to seventh day of the fifth instar were used.

* Parts of this paper were reported at the annual meetings of the Agricultural Chemical Society of Japan held at Kyoto (1958) and Tokyo (1959).

Preparation of Homogenates—As the osmotic pressure of the body fluid of silkworms corresponds to 0.29 to 0.32 *M* (6), 0.35 *M* sucrose solution was chosen as a fractionating medium. The posterior silklands collected were weighed, homogenized in three volumes of 0.35 *M* sucrose with a Potter-Elvehjem-type glass homogenizer (7), and centrifuged at $1,000 \times g$ for 10 minutes. The residue floating at the top of the solution was again homogenized with the same volume of sucrose solution and centrifuged. At this time the residue was precipitated at the bottom of the tubes and once more treated as above. The three milky supernatant solutions were combined as the silkland homogenate and the white residue at the final centrifugation was designated as fraction R. The fraction R contained large amounts of coagulated fibroin secreted into the cavity of silkland.

Differential Centrifugation of Homogenates—The general procedure of differential centrifugation was as follows:

Mitochondrial fraction (M) was sedimented at $3,900 \times g$ for 10 minutes as a yellow and slightly fluffy pellet with a No. 1 rotor of the Marusan refrigerated centrifuge, type 50 B. From the supernatant was obtained large microsomal fraction (L) by centrifuging at $15,000 \times g$ for 20 minutes as a gelatinous pellet consisting of a small amount of yellow layer at the bottom and a translucent one on it with a RP 40 rotor of the Hitachi preparative ultracentrifuge. Then small microsomal fraction (S) was sedimented from the supernatant at $105,000 \times g$ for 60 minutes as a gelatinous and translucent pellet. Supernatants at each centrifugation were removed by decantation and each sediment gave a milky suspension upon homogenizing in the sucrose solution.

Injection of Glycine-1- C^{14} —Each silkworm was injected with glycine-1- C^{14} in 0.05 ml. of physiological saline. The posterior silklands were excised, homogenized and fractionated as above. Each fraction was treated

with 6% perchloric acid, ethanol, ethanol-ether and ether to remove nucleic acids and lipids according to Siekevitz (8), and dried up *in vacuo*. Radioactivities of the dried protein samples were counted with a Geiger-Müller Counter, Aloka JRC Model DC-1C, attached with the SC-1 automatic sample changer. Glycine-1- C^{14} was obtained from the Radiochemical Center, Amersham, England and had a specific activity of 0.1 mc per 2.2 mg.

Methods of Assays—Succinic dehydrogenase was assayed spectrophotometrically at about 23°C (9). Cytochrome c used was prepared from beef heart and purified chromatographically as described by Hagi-hara *et al.* (10). Total nitrogen was determined by semi-micro-Kjeldahl method, inorganic and total phosphorus by Allen's method (11), protein by biuret reaction (12) using ovalbumin as a standard, and RNA phosphorus by orcinol reaction (13) using yeast RNA as a standard. Phospholipids were extracted with chloroform-methanol, purified by contact with pure water overnight (14), and total phosphorus contents were measured. Assay of glycine was performed according to Krueger (15) after 18 hours' hydrolysis of proteins in 6N HCl in sealed tubes at 105°C.

RESULTS

Fractionation of Silk-gland Homogenates—At 15 minutes after injection of glycine-1- C^{14} , the posterior silk glands were homogenized in 9 volumes of 0.35M sucrose and centrifuged stepwise as indicated in Fig. 1 and Table I. The sediments were suspended in 0.35M sucrose and aliquots of these suspensions were used for assays of radioactivity of proteins, succinic dehydrogenase activity, RNA phosphorus, and total nitrogen. Two examples of typical results are presented in Fig. 1 and Table I, respectively.

Fig. 1 shows the glycine-incorporating activity and the succinic dehydrogenase activity of various fractions at different centrifugal forces. The succinic dehydrogenase activity rose with centrifugal force at first and reached a relatively high peak around $2,300 \times g$. On the other hand, the glycine-incorporating activity was low at $2,300 \times g$ and reached a peak at $7,300 \times g$. This result shows that both activities could be separated by differential centrifugation. As shown in Table I, it is clear that the mitochondrial activity was almost completely sedimented at $3,900 \times g$ for

10 minutes. This sediment was designated as fraction M.

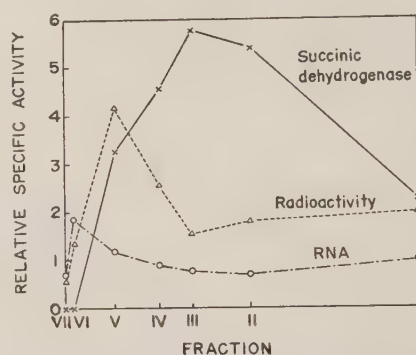


FIG. 1. Glycine-incorporating activity and succinic dehydrogenase activity of various fractions obtained by stepwise centrifugation.

Each silk worm was injected with 10,000 c.p.m. of glycine-1- C^{14} .

The relative specific activity was calculated by dividing the specific activity of each fraction with the specific activity of the homogenate. Specific activity of succinic dehydrogenase, RNA phosphorus, and radioactivity of proteins of the homogenate was 0.105×10^{-7} mole of cytochrome c reduced per minute per mg. nitrogen, 108 μ g. RNA phosphorus per mg. nitrogen, and 17 c.p.m. per mg. protein, respectively.

Positions of fractions along abscissa are plotted in proportion to minimum particle sizes calculated according to Pickel's formula (16), and the conditions of sedimentation were as follows:

$290, 1,100, 2,300, 4,100 \times g$ for 10 minutes, $7,300 \times g$ for 20 minutes, $105,000 \times g$ for 60 minutes and the final supernatant from Fraction I to Fraction VII.

The fraction possessing the highest RNA content came down after separating the fraction of high glycine-incorporating activity (Fig. 1), agreeing with our previous result (4). Hence, the centrifugal separation of the highly incorporating fraction and the RNA-rich fraction was performed at $15,000 \times g$ for 20 minutes in order to increase the yield of the fraction which incorporated glycine-1- C^{14} most actively, and this fraction was named fraction L.

Fraction S possessing the most RNA, was separated from the supernatant of fraction L at the top speed of the ultracentrifuge for 60

TABLE I

Distribution of Radioactivity and Succinic Dehydrogenase in the Silkglad Homogenate

(A) Radioactivity of proteins ¹⁾		(B) Succinic dehydrogenase ²⁾	
Fraction	Specific activity c.p.m./mg. prot.	Fraction	Specific activity mole of cytochrome c reduced/min.
Homogenate	17	Homogenate	9.36×10^{-7}
Sediment at $3,900 \times g$ for 10 min. (Fraction M)	35	Sediment at $3,900 \times g$ for 10 min.	8.86×10^{-7}
Sediment at $7,300 \times g$ for 20 min.	46		
Sediment at $15,000 \times g$ for 20 min. (Fraction L)	29	Sediment at $15,000 \times g$ for 20 min.	0.41×10^{-7}
Sediment at $26,000 \times g$ for 20 min.	17		
Sediment at $105,000 \times g$ for 60 min. (Fraction S)	8	Sediment at $105,000 \times g$ for 60 min.	0
Supernatant	5	Supernatant	0

1) Each silkworm was injected with 10,000 c.p.m. of glycine-1-C¹⁴.

2) The values are for 40 ml. of homogenate or equivalent amount of each fraction.

minutes.

Chemical Compositions of the Cellular Fractions—Distributions of total nitrogen, RNA-phosphorus, and phospholipid-phosphorus among the cellular fractions are shown in Table II.

Total nitrogen was recovered most in the final supernatant and next in fraction S, like liver (17). The nitrogen in fraction L occupied about 10 per cent of the homogenate.

The RNA content of the homogenate was considerably great compared with those of other tissues (18), as Denuce reported (19), and the RNA-phosphorus/total nitrogen ratio

corresponded almost to that of liver microsomes (20). Among the cellular fractions, fraction S contained over a half of total RNA in the homogenate and showed an extraordinarily great RNA-phosphorus/total nitrogen ratio, agreeing with our previous data (3). Fraction M and L also had a considerable amount of RNA, though less than fraction S, possessing the RNA-phosphorus/total nitrogen ratios almost corresponding to that of liver microsomes (20). This can be thought to be due to co-existence of mitochondrial and microsomal structures in fraction M, as mitochondria have generally very little RNA (21).

TABLE II

Chemical Compositions of the Silkglad Fractions

Fraction	Total N ¹⁾	RNA-P ¹⁾		RNA protein	Phospholipid-P ¹⁾		Glycine content of proteins ²⁾
		total			total		
	mg.	μ g.	μ g./mg.N		μ g.	μ g./mg.N	μ g./mg. prot.
Homogenate	39.0	2,640	68	0.28	1,160	30	—
Fraction M	5.2	450	87	0.27	470	90	56
Fraction L	4.2	390	93	0.31	357	85	39
Fraction S	11.5	1,650	143	0.42	278	24	33
Supernatant	17.6	500	28	0.08	110	6	32

1) The values are for 40 ml. of homogenate or equivalent amount of each fraction.

2) The glycine content of fraction R proteins was 115 μ g./mg. protein.

While fraction S is considered to correspond to microsomal fractions of other tissues from the view-point of the centrifugal force used to sediment and had the most RNA, phospholipid-phosphorus was recovered mainly in fractions M and L, and fraction S has but a little.

Fraction R, remained unsuspended at homogenization of silkglands, had a very large yield owing to the secreted fibroin. The ratio of the yield to that of the homogenate increased with the time of growth at the fifth instar. At the sixth day this fraction contained about 4 times protein of the homogenate. As the RNA estimations of this fractions were impossible by both the color reaction of ribose with orcinol (13) and the ultraviolet absorption (22) owing to interfering materials, approximate RNA phosphorus content was estimated after solubilizing with deoxycholate and sedimenting ribonucleoprotein (23). Deoxycholate-soluble protein occupied about 8 per cent of total proteins of this fraction and the RNA/protein ratio of fraction R was 0.08.

Time Course of Glycine-1-C¹⁴ Incorporation In Vivo into Proteins of Cellular Fractions—After definite intervals of glycine-1-C¹⁴ injection, the silkglands were excised, homogenized, and fractionated according to the general procedure. Specific radioactivity of proteins of different fractions as a function of time are shown in Fig. 2.

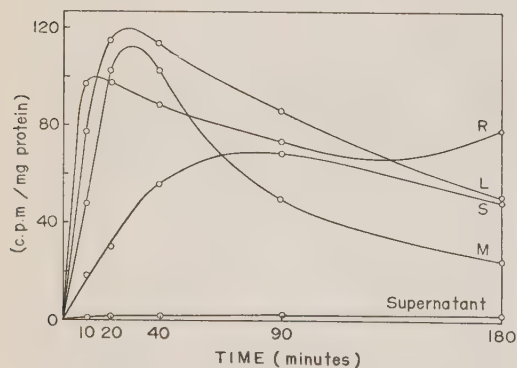


FIG. 2. Time course of *in vivo* incorporation of glycine-1-C¹⁴ into proteins of different fractions of silkgland.

Unlike liver (8), pancreas (24), etc., the radioactivity of the supernatant proteins was very small and the difference of incorporation rates between the particulate proteins and the supernatant proteins was overwhelming. And also there was seen no tendency of later increase of radioactivity of the supernatant proteins.

Among the sedimentable fractions fraction S was less radioactive and reached more slowly to the peak than the others, and fraction L was the most radioactive, agreeing with our previous result (3). The high radioactivity of fraction M is thought to be largely due to the co-existence of large microsomal materials, owing to the insufficient efficiency of separation, as understandable from Fig. 1 and Table II. This situation can be further indicated by hydrolase (acid phosphatase and ribonuclease) distribution measurement and electron microscopy*.

DISCUSSION

Microsomes are generally sedimented by high speed centrifugation from the mitochondrial supernatant and have large amounts of RNA, almost half of the total RNA in the homogenate (17). In cases of highly differentiated tissues, they contain also much phospholipids. Table II shows that fractions L and S had high RNA-phosphorus/total nitrogen ratios corresponding to or surpassing that of liver microsomes and fraction L was rich in phospholipids. The data may mean that the two fractions are microsomal in nature.

Results shown in Table I and Fig. 2 indicate that the glycine incorporation into fraction M proteins was considerably great and the difference between fraction M and L was not so clear as that between mitochondria and microsomes in liver (8). While mitochondria have little RNA in general, fraction M had much RNA corresponding to fraction L, and this may indicate the presence of large amounts of microsomal materi-

* The results of these observations will be shown in the succeeding paper.

als in the fraction besides mitochondria. On the other hand, Fig. 1 indicates that the positions of the highest mitochondrial activity and the highest incorporation activity are different and that the radioactivity of proteins of more readily sedimented fractions with high succinic dehydrogenase activity was comparatively small. These facts may be interpreted as indicating that the mitochondrial proteins in fraction M did not directly concern the high radioactivity of the protein of fraction M like other secretory tissues (26). Above $3,900\times g$ the activities of succinic dehydrogenase and glycine incorporation were overlapping (Fig. 1). The situation that the separation of microsomes from mitochondria could not be performed effectively is a characteristic of the silkgland and may be due to the presence of large microsomal vesicles. Hence the previous results (3) that both succinoxidase and glycine incorporation were the most active in the large granule fraction can be explained by the co-existence of mitochondrial and microsomal structures.

Fraction R, the residual fraction remained not suspended when silkglands were homogenized with a Potter-Elvehjem homogenizer, was the fraction obtained owing to the coagulation of the fibroin in the cavity of silkgland. The initial rise in radioactivity of fraction R, however, was not due to the secreted fibroin, but due to the materials in-

cluded in the coagulated fibroin. Thus it is highly probable that glycine-1- C^{14} was incorporated initially into the microsomal materials in fraction R, considering together with the fact that this fraction contained deoxycholate-

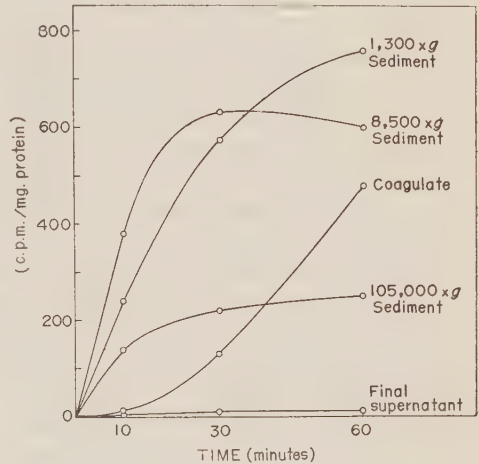


FIG. 3. Incorporation of glycine-1- C^{14} into proteins of cellular fractions obtained by means of the Waring blender homogenization.

1. Each silkworm was injected with 27,000 c.p.m. of glycine-1- C^{14} .

2. Silkglands were homogenized in 9 volumes of 0.25 M sucrose with a Waring blender. Coagulates attached to the blades of the blender were removed and the homogenates were fractionated centrifugally as indicated. Radioactivities of proteins were counted with the type PC-7 Geiger-Müller counter, Kobe Kogyo Corp.

TABLE III

Properties of the Fractions Obtained with the Waring Blender

Fraction	Total N ¹⁾	RNA-P	Phospho-lipid-P	Succin-oxidase	Glycine content
	mg.	$\mu\text{g.}/\text{mg. N}$	$\mu\text{g.}/\text{mg. N}$	$\text{QO}_2/\text{mg. N}$	% ²⁾
Homogenate	23.7	100	—	58	—
Sediment at $1,300\times g$ for 10 min.	8.7	74	37	86	33.2
Sediment at $8,500\times g$ for 10 min.	1.7	68	73	330	23.5
Sediment at $105,000\times g$ for 60 min.	6.2	184	40	18	13.8
Final supernatant	7.2	44	2		4.2
Coagulate	—	0.06	2	—	41.7

Homogenization and fractionation were the same as Fig. 3.

1) The values are for 20 ml. of homogenate or equivalent amount of each fraction.

2) Glycine contents are expressed as percentage of amino acid nitrogen in total nitrogen.

soluble proteins (Table III) and the RNA could be recovered by ultracentrifuging in the form of ribonucleoprotein (23).

On the other hand, when silk glands were blended at high speed with the Waring blender, there were obtained a coagulated protein fraction (Fraction C in Fig. 3) attached to the blades of the blender and a homogeneous suspension of cellular particulates. The yield of fraction C increased with the growth at the fifth instar but always did not exceed that of the suspension.

In Tables II and III it is shown that the lighter microsomal fractions contained much RNA and a little phospholipids, while the heavier microsomal fractions had moderate RNA and much phospholipids. In liver it was reported that readily sedimentable microsomes are comparatively poor in RNA and rich in phospholipids in contrast to less sedimentable microsomes (27, 28). The correlation between RNA and phospholipid contents are not parallel in pancreas (28). Siekevitz and Palade (29) showed that when pancreas microsomes are successively sedimented by ultracentrifugation, leucine-1-C¹⁴ incorporation into their proteins became weaker and the turnover rates became less with the increase of centrifugal force used. The present results shown in Figs. 2 and 3 are similar to this. Thus, the differences of glycine incorporation and chemical contents among the particulate fractions are able to be interpreted to show the heterogeneity of silk gland microsomes, because the silk gland has extraordinarily voluminous endoplasmic reticulum and microsomes are derived mainly from the endoplasmic reticulum (28).

It is noteworthy that glycine-1-C¹⁴ incorporated overwhelmingly into all particulate proteins and very little into supernatant proteins (Figs. 2 and 3). This phenomenon is a characteristic of the silk gland which synthesizes very coagulable fibroin. Considering the fact that only physiological function of the silk gland is rapid synthesis of fibroin, together with the conclusion of above discussion, we can conclude that the microsomes contained in each particulate fractions direct-

ly concern the fibroin synthesis. Of course, this hypothesis must be further confirmed by the existence of the characteristic structures of microsomes, *i.e.*, membrane structures and Palade particles in these fractions and the isolation of fibroin-like proteins and/or peptides. These points will be investigated in succeeding papers.

SUMMARY

The investigation on the *in vivo* incorporation of glycine-1-C¹⁴ into particulate fraction of the posterior silk gland of *Bombyx mori* was developed.

1. By successive differential centrifugation using 0.35 M sucrose, the large granule fraction reported previously (3) was shown to be separated into two particles, *i.e.*, mitochondrial and large microsomal particles.

2. Using a new fractionation procedure, unsuspended fraction (R), sedimentable three fractions (M, L and S), and supernatant fraction were obtained. Succinic dehydrogenase activity in the homogenate was exclusively recovered in fraction M.

3. Glycine-1-C¹⁴ was incorporated overwhelmingly into proteins of fraction R and sedimentable fractions, and little into supernatant proteins. Among the sediments lighter fraction S was less active in incorporation and very rich in RNA, while heavier fractions M and L were most active in incorporation, and rich in phospholipids and RNA though less than fraction S. Fraction R contained large amounts of secreted fibroin in the cavity of silk gland and showed rapid initial turnover of radioactivity.

4. It was concluded that the microsomal structures contained in the sedimentable fractions and also fraction R are the sites of fibroin biosynthesis and that there is diverse heterogeneity in silk gland microsomes.

We wish to thank Mr. K. Noguchi, the Experimental Sericultural Station of Miyagi, for his kindness in supplying us with the silkworm during the course of this investigation and Mr. K. Otomo in this laboratory for his valuable assistance in feeding the silkworm.

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The Activation of Glutaric Acid by an Extract From *Pseudomonas* Bacteria

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There have been a number of studies showing that glutaric acid is derived from lysine and tryptophan in bacteria as well as in animals (1, 2, 3). The further metabolism of this acid in intact animals was reported to result in the formation of α -ketoglutaric acid, but whether this latter is formed directly (4) or *via* the tricarboxylic acid cycle (5) is unknown. Recently Rothstein and Greenberg reported the formation of β -hydroxyglutarate from glutaric acid by rat liver mitochondria (6). Our studies on lysine metabolism by bacterial enzymes (2, 7, 8) led us to investigate the metabolism of glutaric acid.

The present paper reports on the enzymatic formation of the Coenzyme A ester of glutaric acid by the extract of *Pseudomonas* bacteria.

The activating enzyme was prepared from the same strain of *Pseudomonas* which was used for our previous work (7). The composition of the growth medium was as follows (per cent): glutarate 0.35, NH_4Cl 1.0, KH_2PO_4 0.05, K_2HPO_4 0.15, MgSO_4 0.02, yeast extract 0.05, and casamino acid 0.05. The cultures were maintained at pH 7.4 at 30°C with shaking. The cells were harvested, ground with alumina and extracted with 0.1% KCl solution. The extract was centrifuged at $105,000 \times g$ for 30 minutes. The supernatant was treated with protamine sulfate. The clear supernatant was fractionated with solid ammonium sulfate. The fraction between 40 and 60 per cent saturation was dialyzed against 0.005 *M* phosphate buffer (pH 7.4) for 3 hours. The dialysate showed about a 5 fold increase in specific activity over the

original extract. The enzymatic activity was measured spectrophotometrically by hydroxamic acid formation (9). The content of the reaction mixture was shown in Table I.

The activation of glutaric acid is completely dependent upon the presence of ATP as shown in Table I, and also the removal of Co A decreases the activity markedly. Addition of Mg^{++} and glutathione had little

TABLE I
Cofactor Requirements for Glutarate Activation

Reaction system	Activity $\mu\text{mole}/30 \text{ min.}$
Complete system	1.65
„ (Boiled enzyme)	0.00
–ATP	0.00
–Co A	0.58
– Mg^{++}	1.35
–GSH	1.60
– Mg^{++} (+ Mn^{++})	1.82

The complete system contained (in μmoles): glutarate 200, Mg^{++} 10, glutathione (GSH) 10, adenosine triphosphate (ATP) 30, Coenzyme A (Co A) 0.1, Tris buffer (pH 7.0) 120, NH_2OH 500, and enzyme (8.3 mg.) and the final volume was adjusted to 3 ml. with distilled water. The incubation was carried out at 37°C for 30 minutes.

effect on the enzyme activity. The optimal pH was found to be 7.0 and the activity decreased gradually on both sides of this pH. With regard to substrate specificity, the enzyme showed rather higher activity with malonic, succinic and butyric acid than with glutaric acid. The enzyme preparation from cells grown in glucose rather than in gluta-

rate as the sole carbon source showed activity only with succinic acid among the above mentioned acids tested.

The reaction product was identified by paper chromatography by the method of Takeda and Nakagawa (10). The R_f values of glutarylhydroxamate in two solvents were as follows; (1) phenol saturated with H_2O ($R_f=0.40$), (2) isobutyric acid saturated with H_2O ($R_f=0.32$). These R_f values coincide with those for synthetic glutarylhydroxamate and are very different from those of succinyl- and acetylhydroxamate. From the results mentioned above and the results in animals reported by Stern *et al.* (11), we conclude that glutarate is activated by the formation of glutaryl-Co A.

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The Quantitative Determination of Uridine Diphosphoglucuronic Acid*

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For the quantitative determination of uridine diphosphoglucuronic acid (UDPGA) the method of Levvy and Storey (1) is now being used (2), which measures the rate of *o*-aminophenyl glucuronide synthesis by a mouse liver slice, but this method is both troublesome and time-consuming. In 1957, Strominger *et al.* (3) reported that the amount of UDPGA could be estimated through the formation of phenolphthalein glucuronide, which was determined from the decrease in optical density at 540 m μ after incubation of phenolphthalein, UDPGA, and guinea pig microsomes.

This method is more convenient, but it was found necessary to centrifuge off microsomes after color development and moreover not applicable for samples which contained a large amount of protein. TCA and Somogyi method (4) were not suitable for deproteinization in this case, as phenolphthalein was absorbed by precipitated proteins.

The author's experiments indicated that it is more favorable to carry out simultaneous deproteinization and extraction of free phenolphthalein by treatment with chloroform and the chloroform layer then reextracted with glycine buffer. This method did not need centrifugation and at the same time yellowish color originating from proteins or microsomes did not interfere in the course of the assay. This method could also be applied to the

assay on a large amount of sample, and the

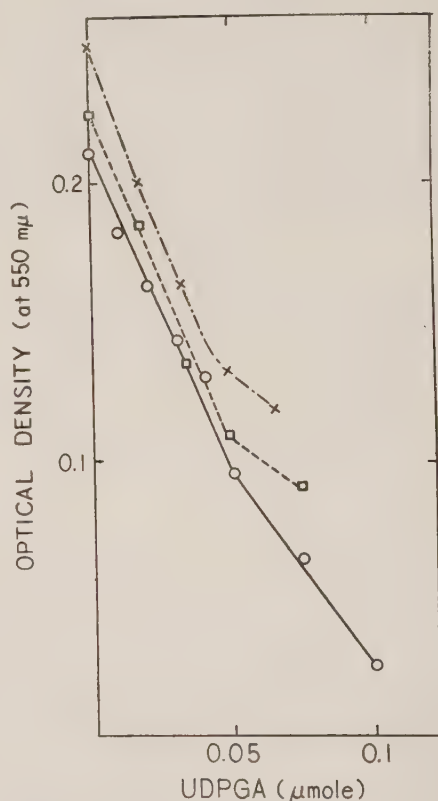


FIG. 1. Calibration curve

Condition was the same as described in the text with the exception that the reaction mixture did not contain potassium saccharate and contained 0.0927 μ mole of phenolphthalein instead of 0.1 μ mole.

- amount of microsomes 0.3 ml. (300 mg. wet liver)
- amount of microsomes 0.2 ml. (200 mg. wet liver)
- ×--- amount of microsomes 0.1 ml. (100 mg. wet liver)

* The following abbreviations are used in this paper: TCA, trichloroacetic acid; UDP-glucose, uridine diphosphoglucose; UDPGA, uridine diphosphoglucuronic acid.

accuracy was increased in this method by the pH value of the sample was kept constant 10.6 for photometric determination. The calibration curve is shown in Fig. 1.

Microsomes obtained from 100 mg. of wet liver would be adequate for this assay, because the excess of microsomes would cause the adsorption of phenolphthalein and its too small amount would decrease the enzyme activity of glucuronide formation. In one hour incubation almost all the UDPGA added was conjugated with phenolphthalein.

This method was not influenced by the coexistence of a large amount of UDP-glucose, glucuronic acid 1-phosphate, or glucuronic acid. Addition of potassium saccharate, which is a specific inhibitor of β -glucuronidase, was able to eliminate the interference by the coexistence of phenolphthalein monoglucuronide in this procedure. The recovery test of UDPGA is shown in Table I. The recovery ratio was 110 per cent and the standard de-

0.1 *M* phosphate buffer (pH 7.5), 0.1 ml. of microsomes obtained from 100 mg. of guinea pig wet liver, and the sample, was made the total volume to 1–2 ml. After incubation at 37°C for one hour, 3 ml. of chloroform was added, the mixture was shaken, and 2 ml. of chloroform layer was removed to another tube after 30 minutes. Three ml. of 1 *M* glycine buffer (pH 10.6) was added to the chloroform solution, the mixture was shaken, and the optical density (*A*) of the water layer was measured at 550 m μ . As a blank, the optical density (*B*) was measured with the same mixture without incubation. The amount of UDPGA was calculated by the following equation:

$$\frac{B-A}{B} \times (\text{amount of phenolphthalein added, } \mu\text{mole}) = (\text{amount of UDPGA, } \mu\text{mole})$$

The author wishes to express his gratitude to Prof. N. Shimazono and Dr. Y. Mano for their helpful advices and continuous encouragement.

TABLE I
Recovery Test

Amount of UDPGA added (μ mole)	0.0163	0.0163	0.0326	0.0326
Amount of UDPGA measured (μ mole)	0.0190	0.0170	0.0400	0.0350
Protein added	guinea pig liver supernatant (0.3 g. wet liver)	rat liver extract (0.25 g. wet liver)	boiled guinea pig liver supernatant (0.6 g. wet liver)	guinea pig liver supernatant (0.6 g. wet liver)

viation was 12.8 per cent. If the sample did not contain protein, the standard deviation was 2.5 per cent.

The following condition seemed to be the most suitable for determination of UDPGA. The reaction mixture containing phenolphthalein (0.1 μ mole) dissolved in 0.3 ml. of 0.001 *M* sodium hydroxide, 0.1 ml. of 0.1 *M* MgCl₂, 0.1 ml. of 0.001 *M* potassium saccharate, 0.2 ml. of

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Preparation of C¹⁴-Labeled Protoporphyrin by Hemolyzed Duck Erythrocytes

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It has been reported that hemolyzed duck erythrocytes convert δ -aminolevulinic acid (ALA) to porphyrins (1). From C¹⁴-labeled ALA as a starting material, C¹⁴-labeled protoporphyrin with high specific activity was obtained using duck erythrocyte hemolysate.

Hemolysate was prepared as follows. The fresh heparinized blood of duck was centrifuged and red cells were washed twice with 0.9% NaCl. The packed cells were hemolyzed by the addition of two volumes of distilled water at 2°C, and the hemolysate was brought back to isotonicity with hypertonic KCl.

To 20 ml. of the hemolysate, 4.8 μ moles (0.6 μ moles of 'porphyrin equivalent') of C¹⁴-ALA (specific activity 2.6 mc per mmole. Total count, 14,000,000 c.p.m.) in 1 ml. of isotonic KCl was added. Incubation was carried out at 37°C for 3 hours and porphyrins were extracted with 10 volumes of ethyl acetate-acetic acid (3:1). Fractionation and determination of porphyrins were made according to the method of Dresel and Falk (2). The amounts of porphyrins thus obtained and the recovery of radioactivity were shown in Table I.

The protoporphyrin fraction was extracted from the HCl solution to ether, the ether solution was washed with water. This was spotted and chromatographed on Whatman no. 1 paper with 2,4-lutidine-water (100:1 v/v) as the solvent. The fluorescent spot which had the R_f value as authentic protoporphyrin was cut out and eluted with 10% HCl. About 65 per cent of the radioactivity applied to the paper was recovered in this eluate. The protoporphyrin thus obtained

was esterified by standing for 15 hours in HCl-methanol at 10°C. The methyl ester was

TABLE I
Radioactive Porphyrins Recovered¹⁾

	assayed photometrically		radioactivity recovered	
	μ mol.	per cent ²⁾	count per min.	per cent
protoporphyrin	0.31	51	4,900,000	35
coproporphyrin	0.0036	0.60	65,000	0.46
uroporphyrin	0.022	3.6	—	—

1) Incubation mixture and conditions: see text.

2) 'Porphyrin equivalent' of the added ALA was taken as 100 per cent.

subjected to paper chromatography in a kerosene-*n*-propanol system (5:1 v/v). Only one spot was detected on the chromatogram and its R_f value was the same as that of pure reference protoporphyrin dimethylester.

The incubation period required to obtain maximum yield was 3 hours under the above conditions. There was little effect of the addition of *o*-phenanthroline as a chelating agent for Fe⁺⁺.

Attempts were also made to prepare porphyrin from glycine. Hemolysate were incubated with glycine, succinate, and ATP or yeast concentrate, but these gave only poor results.

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LETTERS TO THE EDITORS

The Journal of Biochemistry, Vol. 49, No. 1, 1961

Amino Acid Activating Enzyme Free from Pyrophosphate Exchange

The preceding papers (1, 2) have postulated that there are at least two enzyme fractions involved in the incorporation of C^{14} -glycine into the protein of the particulate fraction (RD) of posterior silk gland, and that one may concern with activation of the amino acid, and the other with incorporation of activated amino acid into the particulate fraction. The former has been shown, furthermore, to possess the activity in catalyzing glycine dependent exchange of PP^{32} with ATP, but no activity in formation of glycine hydroxamate.

This report is concerned with a description of further purified preparation of the amino acid activating enzyme. The purified preparation stimulated the incorporation of C^{14} -glycine into the particulate fraction in the presence of ATP and the incorporation enzyme (E_2 or E_{21}), but its activity for pyrophosphate exchange was almost lost.

Cellular fractions (RD, E_2 , E_{21} , E_3 , N_8 and N_6 fractions) were prepared from posterior silk gland of *Bombyx mori* by the method described previously (1, 2). The precipitate of the crude activating enzyme (N_8) was dissolved in a buffered medium (1) (pH 8.0). To the solution powdered ammonium sulfate was added to attain 60 per cent saturation. After standing for one hour, the precipitate was centrifuged off and discarded. The supernatant was then brought to 80 per cent saturation by the further addition of ammonium sulfate. The precipitate was collected, dissolved in the buffered medium and denoted as N_{88} . The solution of N_{88} was again adjusted to pH 4.5 with 0.2M acetic acid and the resulting precipitate was removed by centrifugation. The supernatant was then brought to 80 per cent saturation by addition of powdered ammonium sulfate. The precipitate was collected, redissolved, and denoted as N_{888} . The reaction conditions for the in-

corporations of C^{14} -glycine and C^{14} -alanine into the particulate protein were same as described previously (1, 2).

TABLE I

Comparison of Enzymic Activities for Glycine Dependent iPP Exchange and Incorporation of C^{14} -glycine

Enzyme fraction	iPP exchange ¹⁾	Incorporation system ²⁾	c.p.m. per mg. of protein	
			exp. 1	exp. 2
E_3 ³⁾	61.1	$RD^{5)} + E_{21}^{6)} + E_3$	45	
N_{88} ⁴⁾	50.2	$RD + E_{21} + N_{88}$	142	117
N_{888} ⁴⁾	1.5	$RD + E_{21} + N_{888}$	135	
		$RD + E_{21}$		5

1) Per cent exchange per mg. of protein.

2) The reaction systems consisted of 10 μ moles of ATP, 5 μ moles of $MgCl_2$, 50 μ moles of Tris-HCl (pH 8.0), 0.025 μ moles glycine-1- C^{14} (100,000 c.p.m.), 400 μ moles of sucrose, 50 μ moles of KCl, an amino acid mixture containing 19 amino acids except glycine, 0.2 ml of RD (1 to 1.5 mg. of protein), 0.15 ml. of E_{21} (1 mg. of protein) and 0.1 ml of N_{88} or N_{888} (0.15 mg. of protein). These systems were incubated at 37°C for 30 minutes.

3) Supernatant obtained after precipitation of E_2 at pH 4.9.

4) Enzyme fraction prepared from N_8 as described in the text.

5) Deoxycholate-treated particulate fraction of posterior silk gland of *Bombyx mori*.

6) Reprecipitated fraction from E_2 at pH 5.2.

As shown in Table I, the activity of the N_8 fraction for catalyzing glycine dependent iPP exchange diminished markedly on the process of the purification. On the other hand, the ability for the incorporation of C^{14} -glycine was practically unchanged. The activity of N_{88} for incorporation of C^{14} -glycine was completely destroyed by heating at 95°C for 3 minutes. N_{88} kept its activity of incorporation unchanged for three days when stored at -10°C, but the activity of iPP exchange reduced to one fiftieth of the fresh preparation on storage under the same condi-

tion (Table II). Furthermore, it could be seen from Table III that the incorporation

TABLE II

Stability of N_{88} for Glycine Dependent iPP Exchange and Incorporation of C^{14} -glycine

Enzyme fraction	iPP exchange	Incorporation system	c.p.m. per mg. of protein
N_{88}	50.2	$RD + E_{21} + N_{88}$	142
$N_{88A}^{1)}$	1.0	$RD + E_{21} + N_{88A}$	127

1) N_{88} preparation stored frozen at $-10^{\circ}C$ for three days.

TABLE III

Comparison of Enzymic Activities for Alanine Dependent iPP Exchange and Incorporation of C^{14} -alanine

Enzyme fraction	iPP exchange	Incorporation system	c.p.m. per mg. of protein
N_6	25.1	$TRD^{1)} + E_{21} + N_6$	65
N_{88}	1.5	$TRD + E_{21} + N_{88}$	139

1) Deoxycholate-treated particulate fraction of posterior silk gland of *Attacus ricini*.

of C^{14} -alanine into the protein of particulate fraction (TRD) from posterior silk gland of *Attacus ricini* was stimulated more with N_{88} which had no detectable activity toward the alanine dependent exchange of iPP than

with N_6 which showed marked iPP exchange activity.

These findings suggest that the amino acid dependent exchange reaction may be not associated with incorporation of C^{14} -amino acid into the particulate protein of posterior silk gland and that, therefore, amino acids may be activated not by way of aminoacyl adenylate postulated by Hoagland (3) and Berg (4), but by other pathway for amino acid incorporation into peptide linkage. Recent studies by Beljansky (5) indicated that his incorporation enzyme (6) could possibly catalyze some new kind of amino acid activation. Thus further study will be needed to clarify the occurrence of an alternative mechanism for amino acid activation.

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LETTERS TO THE EDITORS

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Application of Hexanol-Celite Column for an Assay of Inorganic Phosphate

For the separation of inorganic phosphate (*iP*) from other phosphate compounds such as ATP, etc., isobutanol (1) or isobutanol-benzene (2) extraction is widely used. Recently, Hagihara and Lardy (3) devised an excellent method of removing *iP* from a mixture of phosphate compounds. To study the *iP* uptake in a mitochondrial system during oxidative phosphorylation, they used hexanol or heptanol-2 and siliconized Celite in a small column. iP^{32} in a reaction mixture is completely trapped in the column and uptake of the inorganic phosphate is easily known by counting the radioactivity of the effluent.

As their method is simple and particularly advantageous in that it permits large numbers of samples to be run simultaneously, we have applied their method for an assay of *iP* by colorimetry.

Column—Hand column is almost the same as that of Hagihara-Lardy. To speed up the assay, a larger glass tube was used (inside diameter 0.8 cm.). For ordinary use (0.01 to 0.5 μ moles *iP*), the height of the packed Celite is 1 cm. or less. In the method of Hagihara-Lardy, special care was paid for the volume change of the effluent, because the radioactivity of the effluent was compared with that of the reaction mixture. In our case, however, all treatments become simpler, since only the total *iP* is in question.

Separation—By adding one volume of reaction mixture to two volumes of cooled Reagent A (3) (0.9 *M* $HClO_4$, 0.6 *M* Na_2SO_4), the reaction is stopped and the mixture is deproteinized. Then, three volumes of Reagent B (3) (a mixture of 0.5 volumes 4% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.4 volume of methanol, 0.012 volume of hexanol and water (about 0.15 volume) to make the final volume 1.) are added to form phosphomolybdic acid

(PMA). After standing for a few minutes, the solution is poured into the column. A yellow band of PMA is formed on the upper part of the Celite and the separation is completed. The column is kept in a refrigerator during the percolation (usually 10 to 20 minutes).

Washing—When all of the solution has passed through, the column is washed with 1 ml. of a mixture of water, Reagent A, and Reagent B without containing molybdate (volume ratio 1:2:3). This process is necessary because traces of the reaction mixture and excess molybdate are retained in the column. By this washing procedure, the subsequent reduction is little affected by salts or other substances in the reaction mixture.

Elution and Reduction—PMA which was trapped in the column is eluted by 2 ml. of a mixture of ethanol, hexanol and H_2SO_4 (80:20:5). The effluent is taken in a graduated test tube and enough eluent is added to make a total volume 5 ml. Then 0.05 ml. of a reducing reagent ($SnCl_2$ 1 g in HCl 2.5 ml. was diluted 20 times with 5% H_2SO_4 before use) is added. Molybdenium blue produced by the reduction is measured by E_{720} . Absorbance is very stable.

As no solvent extraction process is present in this method, total *iP* can be used without any loss, and sensitivity is increased. The elution and reduction can also be done in an aqueous phase. Full details will be reported elsewhere.

Many thanks are due to Prof. Hagihara of Osaka University for his constant instructions and advices.

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